

Mitotic Mechanics and Mechanisms of the Budding Yeast *Saccharomyces cerevisiae*

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ABSTRACT

DAVID CANNON BOUCK: Mitotic Mechanics and Mechanisms of the Budding Yeast
Saccharomyces cerevisiae

(Under the direction of Dr. Kerry Bloom)

Successful cell division requires the equal segregation of the replicated genome. This process is carried out by a cellular machine known as the mitotic spindle, which is largely conserved across eukaryotes. I have used the budding yeast *Saccharomyces cerevisiae* as a model organism to better understand the mechanics of the mitotic spindle and the mechanisms that coordinate events during mitosis.

Prior to segregation, sister chromatids form bi-polar attachments to microtubules nucleated from the two spindle pole bodies. These attachments are mediated by the kinetochores which link centromeric DNA to microtubules. Biorientation of sister chromatids results in their alignment at the spindle equator, a state known as metaphase. During metaphase, spindle length remains stable. This stability has been largely attributed to the activity of microtubule motors in the spindle. I have taken an alternative approach to dissecting the forces within the metaphase spindle by examining the role of chromatin structure of sister chromatids. By lowering chromatin packaging, I have shown that spindle length is directly regulated by the stretching of pericentric chromatin. This result demonstrates that chromatin is an important structural member of the metaphase spindle.

Following metaphase, chromosomes segregate and the cell undergoes cell division to produce two daughter cells. I found that during anaphase, a fraction of inner kinetochore proteins re-localizes to the spindle midzone. This re-localization requires the activity of a yet unidentified motor. Mutation of the inner kinetochore protein Ndc10p results in defects in spindle stability and cell division. This mechanism of spatial regulation of kinetochore proteins appears to contribute to the coordination of chromosome segregation, spindle elongation, and cell division.

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LIST OF ABBREVIATIONS AND SYMBOLS

α -factor	alpha factor
bp	base pair
CBF3	centromere binding factor 3
CEN	centromere
CFP	cyan fluorescent protein
ChIP	chromatin immunoprecipitation
Δ	delta (gene deletion)
Dex	dextrose
DNA	deoxyribonucleic acid
FLIP	fluorescence lost in photobleaching
FRAP	fluorescence recovery after photobleaching
G1	gap one
G2	gap two
Gal	galactose
GFP	green fluorescent protein
h	hours
IBS	Ipl1p-Bir1p-Sli15p
INCENP	inner centromere protein
kb	kilobase pairs
lacI	lactose repressor
lacO	lactose operator
M	mitotic phase

MAPs	microtubule associated proteins
μg	micrograms
min	minutes
ml	milliliters
μm	micrometers
mM	millimolar
NA	numerical aperature
nm	nanometer
PCR	polymerase chain reaction
pN	piconewton
RFP	red fluorescent protein
S	synthesis phase
SD	standard deviation
sec	seconds
SPB	spindle pole body
U	enzymatic units
YFP	yellow fluorescent protein
YPD	yeast peptone dextrose
YPG	yeast peptone galactose

CHAPTER ONE

GENERAL INTRODUCTION

Overview

As the cell defines the basic unit of life, it follows that cell division is among the most important processes of life. Cell division allows for the replication of single-celled organisms and creates the mass of cells required for the complex systems of multi-cellular organisms. Thus, for all organisms, accurate cell division is essential to the propagation of the species.

The life of a cell has been described in terms of a cell cycle, consisting of a series of cellular events executed in a specific order (Johnson and Rao, 1970; Rao and Johnson, 1970). The budding yeast *Saccharomyces cerevisiae* has proven a valuable model organism for the study of all stages of the cell cycle (Hartwell, 1991; Nasmyth, 1996). Budding yeast live as either haploid or diploid cells, possessing 16 or 32 chromosomes, respectively. In the G1 phase of the cell cycle, cells are unbudded. Commitment to cell division occurs at the G1/S transition known as START (Hartwell et al., 1974). START initiates three separate, parallel pathways: bud formation, DNA replication, and spindle pole body (centrosome) duplication (Hartwell et al., 1974). S phase cells are apparent by their small bud size. While the genome is replicated, the bud continues to grow and duplicated spindle pole bodies separate.

Transition from S phase to G2/M is characterized by the completion of DNA replication, formation of a bipolar spindle, and the attachment of sister chromatids to the mitotic spindle. Sister chromatids can become attached to the spindle prior to the completion of DNA replication due to the close proximity of centromeres to early-firing origins of replication, suggesting that S phase and M phase may partially overlap in normally dividing cells (Goshima and Yanagida, 2000; McCarroll and Fangman, 1988).

Completion of mitosis is marked by the equal segregation of sister chromatids to the daughter cells. Cytokinesis follows, separating the cytoplasm into two discrete compartments. In budding yeast, cell division is complete when cell abscission, dissolution of cell wall material joining the cells, is completed (Kuranda and Robbins, 1991). The newly formed cells may remain quiescent, or given favorable growth conditions, enter into another cell division cycle.

This introduction will focus on the mitotic stage of the cell cycle, with an emphasis on two areas of cell division research. First, the mechanics of the mitotic spindle will be reviewed, including an inventory of spindle components and how they function during metaphase and anaphase. Second, this introduction will focus on a mitotic mechanism, namely the relocation of centromere-associated proteins to the spindle midzone in late anaphase, which contributes to the completion of the cell division cycle. Subsequent chapters will discuss the work that I have performed to increase understanding of both the mechanics and mechanisms of mitosis using budding yeast.

Microtubules

The budding yeast spindle contains two microtubule organizing centers known as spindle pole bodies (Byers and Goetsch, 1975; Winey and Byers, 1993). As yeast carry out a closed mitosis (no nuclear envelope breakdown), spindle pole bodies nucleate nuclear and cytoplasmic microtubules which play roles in mitosis specific to their compartmentalization. Cytoplasmic microtubules are critical to the positioning of the nucleus to the future site of cell division (bud neck) (Pearson and Bloom, 2004). Nuclear microtubules are the primary component of the mitotic spindle, creating the structure of the spindle and contributing to the mechanics of the of the chromosome segregation machinery (Inoue, 1953).

Nuclear microtubules are divided into two classes: interpolar and kinetochore microtubules (Winey et al., 1995). Interpolar microtubules do not directly interact with chromosomes, but are essential to the maintenance of spindle structure. Interpolar microtubules nucleated from opposing poles are anti-parallel (opposite polarity), and are regularly spaced from each other, suggesting they are organized by bridging molecules (Winey et al., 1995). Cross-linked interpolar microtubules may be considered the “backbone” of the mitotic spindle. The other class of nuclear microtubules is the kinetochore microtubules. The plus-ends of kinetochore microtubules are associated with a large, multi-protein, centromere-bound complex known as the kinetochore.

Microtubules are polar, dynamic polymers (Inoue and Sato, 1967; Mohri, 1968). Heterodimers of alpha and beta tubulin are added or removed from the polymer, leading to microtubule growth or shortening, respectively (Weisenberg et al., 1968). In budding yeast, the minus-end of microtubules are embedded in the outer plate of the spindle pole body, and microtubule plus-ends are oriented away from spindle pole bodies (O'Toole et

al., 1999). Minus-ends are static, while plus-ends are dynamic in exchange of tubulin subunits (Maddox et al., 2000). Microtubule dynamics can be described in terms of the frequency of rescue and catastrophe events, and the velocity of microtubule growth and shortening (Mitchison and Kirschner, 1984; Walker et al., 1988). The association of microtubule associated proteins and microtubule motor proteins regulates microtubule dynamics (Pearson et al., 2003). Microtubule dynamics are important for the “search and capture” of sister chromatids in early mitosis, and the generation of tension at kinetochores during metaphase, a requirement for the spindle checkpoint (Rieder and Salmon, 1998).

Like most other eukaryotes, the budding yeast mitotic spindle maintains a stable spindle length during metaphase (Goshima et al., 2005; Mitchison et al., 2005). The cross-linking of interpolar microtubules in metaphase spindles could easily provide a mechanism by which a stable spindle length would be maintained. This model suggests that interpolar microtubules would also be stable, while kinetochore microtubules would be dynamic. More recently, however, the dynamics of all nuclear microtubules has been observed in pre-anaphase cells (Higuchi and Uhlmann, 2005). At anaphase onset, Cdc14p is released as part of the FEAR and MEN pathways, and contributes to a number of pathways at the completion of mitosis (D'Amours and Amon, 2004). Cdc14p phosphatase activity is also required for nuclear microtubule stabilization, but the mechanism by which interpolar microtubules contribute to spindle stability when they are themselves dynamic is not clear.

Anaphase A (the shortening of kinetochore microtubules) happens concurrently or shortly after anaphase B (spindle elongation) in budding yeast (Pearson et al., 2001).

This suggests that kinetochore microtubules are only able to depolymerize after anaphase onset, but polymerization is inhibited. In contrast, after anaphase onset, interpolar microtubules do not depolymerize until the spindle has fully elongated (Buvelot et al., 2003). In fact, interpolar microtubules must grow during anaphase to fully separate spindle pole bodies the distance of the mother-bud axis (approximately 10 μm). This difference is likely due to other proteins associated with the plus-ends of these two classes of nuclear microtubules. In the case of kinetochore microtubules, kinetochore (or kinetochore-associated) proteins may allow for or even contribute to depolymerization. At interpolar microtubules, midzone proteins including Ase1p, Slk19p, and passenger proteins (see below) might actually protect plus-ends from depolymerization and promote polymerization. In this case, the primary function of Cdc14p might be to target microtubule stabilizing/growth promoting proteins to the spindle midzone (Pereira and Schiebel, 2003).

Structure of the Inner Centromere

The budding yeast centromere consists of three domains, spanning 125 base pairs (bp), which are conserved across all 16 chromosomes (Fitzgerald-Hayes et al., 1982). The remarkably small size of the centromere, has led to its being referred to as a point centromere, while the centromeres of other eukaryotes are considered regional centromeres, spanning anywhere from a few kilobase pairs (kb) to several megabase pairs (Cleveland et al., 2003).

While the sequence or composition of centromeres in many organisms has been identified, the physical structure and organization of this region of chromatin *in vivo* is

not well understood. Centromeres in all eukaryotes have at least one nucleosome containing a specialized, centromere specific histone H3 variant (Cse4p in budding yeast; CENP-A in mammals) (Meluh et al., 1998; Palmer and Margolis, 1985). This specialized nucleosome appears to be critical in defining the centromere functionally, and in recruiting kinetochore proteins.

The interaction of centromeric DNA with a Cse4p-containing nucleosome has been recently modeled (Bloom et al., 2006). The replacement of H3 with Cse4p results in a significant difference in the histone tails that have the greatest interactions with the DNA. Cse4p tails are predicted to guide centromeric DNA in a specific path as it enters and exits this nucleosome. This path aligns centromere-flanking sequences in close proximity to each other near the centromere.

The enrichment of cohesin, a multi-subunit complex capable of topologically linking two strands of DNA, near the centromere by chromatin immunoprecipitation is difficult to reconcile with the observation that sister chromatid centromeres are separated by approximately 800 nm during metaphase (Blat and Kleckner, 1999; Pearson et al., 2001). This apparent conflict in results can be resolved by considering that cohesin might link individual chromatids to themselves near the centromere (*intrastrand* cohesion), and link chromatids to their sisters at locations further from the centromere (*interstrand* cohesion) (Bloom et al., 2006).

This arrangement of pericentric chromatin allows for a re-defining of the budding yeast centromere. In fact, it may be appropriate to identify the centromere as a 14-20 kb region centered on a conserved 125 bp core sequence. This definition also allows for budding yeast to have a proper inner centromere similar to higher eukaryotes. The

localization of passenger proteins to the inner centromere in mammalian cells and their role in correcting improper microtubule attachments to the kinetochore suggests that the inner centromere might be the site of tension sensing required for satisfaction of the spindle checkpoint (Cooke et al., 1987).

Pericentric chromatin is composed of a highly ordered array of nucleosomes, as assayed by micrococcal nuclease mapping of their position (Bloom and Carbon, 1982). When tension is applied to a sister chromatid pair, pericentric chromatin structure may be altered through the dissociation of nucleosomes, or partial unraveling of DNA around nucleosomes (Pearson et al., 2001). This change in chromatin structure, due to the mechanical strain placed on it, may lead to inactivation of Ipl1p/Aurora B. Alternatively, a lack of strain (tension) in the chromatin would activate Ipl1p/Aurora B to destabilize the microtubule attachment through phosphorylation of outer kinetochore proteins.

Kinetochores

The kinetochore is a large, multi-protein complex that bridges dynamic microtubule plus-ends to the centromeric region of chromosomes. Over 65 proteins have been characterized as kinetochore proteins based on their localization, association with the centromere (whether direct or indirect) as detected by chromatin immunoprecipitation, and association with other known kinetochore proteins (He et al., 2001; McAinsh et al., 2003). In general terms, kinetochore proteins are described as “inner,” “mid,” or “outer.” Inner kinetochore proteins are most closely associated with centromeric DNA, while outer kinetochore proteins are most closely associated with microtubule plus-ends. Further, centromere association dependency has been used to

map the hierarchy of kinetochore proteins with respect to the centromere. For example, the association of mid and outer kinetochore proteins is dependent on functional inner kinetochore proteins. However, inner kinetochore proteins do not require outer proteins for centromere association (as assayed by chromatin immunoprecipitation).

The three classes of kinetochore proteins reflect the three primary functions of the kinetochore (Rieder and Salmon, 1998). First, the kinetochore binds specifically to centromeric DNA. The inner kinetochore protein complex, CBF3, specifically binds the centromere *in vivo* and *in vitro* (Lechner and Carbon, 1991; Russell et al., 1999). Mutations in either CBF3 components or centromeric DNA result in compromised binding (Lechner and Carbon, 1991). This specificity is critical to prevent the formation of ectopic kinetochores, which would prove lethal to the cell. The inner kinetochore also has a specialized nucleosome bearing the centromere-specific H3 variant Cse4p (Meluh et al., 1998). Cse4p may interact with other inner kinetochore proteins to stabilize their binding, and interact with centromeric DNA to influence its path through the kinetochore (Bloom et al., 2006).

The outer kinetochore proteins function to attach the kinetochore to the dynamic plus-end of a microtubule. Recent work has shown that the outer kinetochore complex, DAM/DASH, is able to form rings around microtubules *in vitro* (Miranda et al., 2005a; Westermann et al., 2005). This structure explains how kinetochores maintain stable attachments to dynamic microtubules. Additionally, this ring might affect microtubule dynamics through influencing microtubule rescue frequencies.

The mid kinetochore proteins contribute to the accuracy of proper kinetochore formation and function. Mutations or deletions in genes encoding these proteins result in

increased levels of chromosome loss (Goshima and Yanagida, 2000; Janke et al., 2001; Measday et al., 2002; Ortiz et al., 1999). While the specific functions of most of these proteins are not well understood, it is likely that they interact with the signaling of the spindle checkpoint and the correction of aberrant kinetochore attachments. The Nuf2/Ndc80 complex has been specifically identified as playing roles in these functions (DeLuca et al., 2003; Janke et al., 2001).

Kinetochore formation and regulation

There are likely three mechanisms by which kinetochores might be formed. Models can be divided into centromere-centric and microtubule-centric classes. The centromere-centric model is based on the result that the vast majority of kinetochore proteins are found to associate with the centromere by chromatin immunoprecipitation in nocodazole (a microtubule depolymerizing agent) treated cells, suggesting that the kinetochore can form at the centromere and simply wait to be found by a microtubule. Additionally, Cse4p is incorporated at the centromere in early S phase, the time of centromere replication (Pearson et al., 2004). Thus, the incorporation of Cse4p may lead to the assembly of the remainder of the complex.

While many kinetochore proteins associate with the centromere in a microtubule-independent manner, it is not clear if this is the normally occurring process in cells. In fact, a number of kinetochore proteins have been found to associate with microtubules in a centromere-independent manner, giving rise to a microtubule-centric model for kinetochore assembly (Goh and Kilmartin, 1993; Muller-Reichert et al., 2003; Zeng et al., 1999). Among these proteins are members of the inner kinetochore complex CBF3

(Bouck and Bloom, 2005). CBF3 association with microtubules may reflect a partial or “pre-” kinetochore complex that matures into a complete kinetochore upon centromere binding. Thus, the possibility remains that the kinetochore may assemble on microtubules in a centromere-independent manner. In this case, the centromere would then be found through a “search and capture” mechanism of kinetochore proteins already associated with dynamic microtubules (Tanaka et al., 2005).

A third model explaining kinetochore formation is a compromise of these models. It proposes that the inner kinetochore is assembled at the centromere and the outer kinetochore is assembled at the microtubule. A kinetochore is then formed when these two halves bind each other. This model is supported by data demonstrating how kinetochore attachments are destabilized. Phosphorylation of Dam1p by the Ipl1p (Aurora B) kinase leads to weakened interaction between the DAM/DASH and Ndc80/Nuf2 complexes (Cheeseman et al., 2002; Shang et al., 2003). Accordingly, kinetochore reassembly would require the centromere-associated kinetochore proteins (Ndc80/Nuf2 complex inward) to be found by the microtubule-associated kinetochore proteins (DAM/DASH complex). It is unclear whether this mechanism would be applicable to new attachments as well as corrected attachments.

Kinetochore attachments are only productive if they result in the equal segregation of chromosomes in anaphase. Thus, the cell must recognize attachments that are not amphitelic. In budding yeast, where only one microtubule binds at each kinetochore, the most likely attachment error is syntelic attachment. In other eukaryotes, where multiple microtubules bind a single kinetochore, merotelic attachments may also occur and must be recognized and corrected by the cell.

Proper attachments bear two hallmarks, both of which are necessary to satisfy the spindle checkpoint before a cell will go into anaphase (Lew and Burke, 2003). First, a microtubule must be associated with the kinetochore. Second, that microtubule attachment must generate tension. Sister chromatids having mono-attachments or syntelic attachments will not have tension at their kinetochores. In both cases, the cell cycle is delayed until the error is corrected.

Correction of mono-attachment is carried out by simply delaying anaphase, giving the cell more time to establish attachments at the unattached kinetochore. Syntelic attachments are corrected by Ipl1p-dependent destabilization of the improper attachment. Recently, it was shown that Ipl1-dependent destabilization of kinetochore attachments results in cell cycle delay by the creation of mono-oriented sister chromatid pairs (Pinsky et al., 2006). This result suggests that the spindle checkpoint is primarily sensitive to non-attached kinetochores.

The question of how tension is sensed, presumably by Ipl1p, at a kinetochore attachment remains under active investigation. It has been proposed that Ipl1p is a part of a tension sensing complex with Sli15p (INCENP) and Bir1p (Survivin) (Sandall et al., 2006). If this complex directly senses tension, then it may also position Ipl1p to immediately act when tension is not sensed. One could imagine that mechanical strain applied to this complex might result in the regulation of Ipl1p activity. This regulation could occur through control of kinase activity, or spatial regulation of the complex. Ipl1p kinase activity is attenuated through Sli15p association, suggesting a possible means of regulation (Kang et al., 2001). Additionally, Ipl1p has been reported to dissociate from

the kinetochore upon biorientation, a means by which Ipl1p might be spatially regulated (Tanaka et al., 2002).

Microtubule motors

Budding yeast has 6 kinesin motors (Kip1p, Kip2p, Kip3p, Cin8p, Kar3p, and Smy1p) and one dynein motor (Dhc1p) (Hildebrandt and Hoyt, 2000). All of these, except Smy1p, function during mitosis (Lillie and Brown, 1992). No single kinesin is essential, suggesting functional redundancy among these proteins. Microtubule motors are required for the essential mitotic processes of bipolar spindle formation, spindle positioning, metaphase spindle stability, and anaphase (i.e. spindle elongation). Here the roles of the relevant kinesins (Kip1p, Cin8p, and Kar3p) in the metaphase spindle will be discussed.

Cin8p and Kip1p belong to the BimC/kinesin-5 family of motors. Neither is essential, suggesting redundancy in their functions (Hoyt et al., 1992). The *Drosophila* homolog, KLP61F, forms a homotetrameric complex that allow for the cross-linking of microtubules (Cole et al., 1994). Consistent with this, Cin8p and Kip1 are localized to the spindle where they may act as microtubule cross-linking intermediates. The *Xenopus* homolog Eg5 exhibits plus-end directed motility, suggesting that Cin8 and Kip1p would likewise have plus-end directed motility (Gheber et al., 1999; Sawin et al., 1992).

Kar3p is in the kinesin-14 family of motors, which have the motor domain at the C-terminus. In contrast to the BimC motors, Kar3p forms heterodimers with the accessory proteins Cik1p or Vik1p that exhibit minus-end directed motility (Manning et al., 1999; Middleton and Carbon, 1994). Kar3p-Cik1p functions primarily on

cytoplasmic microtubules during karyogamy. Kar3p-Vik1p localizes to the nucleus where it functions within the mitotic spindle.

The function of Kip1p, Cin8, and Kar3p in the metaphase spindle is best understood through phenotypic analysis of cells lacking or overexpressing these proteins. Cells lacking either Cin8p or Kip1p have shorter spindles, and cells lacking both fail to separate spindle pole bodies to form a bipolar spindle and die (Hoyt et al., 1992). Loss of functional Cin8p and Kip1p in cells arrested in metaphase also results collapse of the spindle (Saunders and Hoyt, 1992). Additionally, overexpression of Cin8p leads to long metaphase spindles (Saunders et al., 1997). Together, these results suggest that Cin8p and Kip1p contribute to outward (elongation) spindle forces.

Spindle collapse in *kip1 Δ cin8 Δ* cells is suppressed by loss of Kar3p function (Saunders and Hoyt, 1992). Additionally, this overexpression of Kar3p results in shorter spindles (Saunders et al., 1997). These results suggest that Kar3p antagonizes the activity of the BimC motors by generating inward (shortening) spindle force.

The combination of these data has led to a motor-centric model of spindle length determination and stability during metaphase (Hildebrandt and Hoyt, 2000). Cin8p and Kip1p generate an outward force that is opposed by a Kar3-dependent inward force. While this model describes the contributions of motors along interpolar microtubules, it ignores the contribution of kinetochore microtubules and sister chromatids to spindle mechanics. Given that sister centromeres are separated in metaphase by kinetochore microtubule-dependent pulling forces, it seems likely that sister chromatids themselves would contribute to the metaphase force balance. In chapter two, a new model for

metaphase spindle regulation, which addresses the contribution of sister chromatids, will be presented.

Coordination of Mitotic Events

Once the metaphase spindle forms attachments to all sister chromatid pairs, the anaphase promoting complex is activated and sister chromatids are segregated (Ciosk et al., 1998). Chromosome segregation involves the shortening of kinetochore microtubules toward the spindle pole (anaphase A) and elongation of the spindle (anaphase B). Following complete spindle elongation, the cell cycle is completed by the physical separation of the cells. In budding yeast, cell division is comprised of two steps: cytokinesis (i.e. separation of the cytoplasm into two compartments) and cell separation (or abscission) which resolves the bud from the mother cell. Cell division must be temporally regulated with respect to anaphase onset in order for cell division to be successful. For instance, cytokinesis prior to anaphase would result in an aploid bud and diploid mother (assuming the spindle is oriented in the mother exclusively). In *Schizosaccharomyces pombe*, this is known as a “cut” phenotype as the spindle (and chromosomes) are cut in half by the cytokinetic machinery (Hirano et al., 1986).

The coordination of mitotic events, particularly during anaphase and cytokinesis, is carried out in part by the passenger proteins that associate with the inner centromere prior to anaphase onset. At anaphase onset, passengers re-localize to the spindle midzone, where they contribute to spindle stability, microtubule regulation, and cytokinesis (Andrews et al., 2003; Buvelot et al., 2003; Carmena and Earnshaw, 2003).

In budding yeast, the passenger proteins Ipl1p (Aurora B), Sli15p (INCENP), and Bir1p (Survivin) also localize to the spindle midzone (Pereira and Schiebel, 2003; Widlund et al., 2006). Loss of Ipl1p results in a delay of spindle disassembly, indicating a role in regulating interpolar microtubules during late anaphase. Loss of Sli15p leads to weakened anaphase spindles, and loss of Bir1p leads to subtle elongation defects during anaphase. Clearly this complex is important in regulating the spindle midzone during late anaphase.

As presented in chapters three and four, the inner kinetochore complex CBF3 also re-localizes from the centromere to the midzone during anaphase. Mutations in the CBF3 component Ndc10p lead to defects in spindle elongation and cell separation, indicating a role for Ndc10p in coordinating these late mitotic events (Bouck and Bloom, 2005). CBF3 interacts with the canonical passenger complex (Ipl1p-Bir1p-Sli15p) through Bir1p, and CBF3 localization to the midzone is likewise dependent on Bir1p (Bouck and Bloom, 2005; Yoon and Carbon, 1999). These data suggest that CBF3 is part of the passenger complex in budding yeast (discussed further in chapter four).

CHAPTER TWO

PERICENTRIC CHROMATIN IS AN ELASTIC COMPONENT OF THE MITOTIC SPINDLE

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Abstract

Prior to chromosome segregation, the mitotic spindle bi-orient and aligns sister chromatids along the metaphase plate. During metaphase, spindle length remains constant, suggesting that spindle forces (inward and outward) are balanced. The contribution of microtubule motors, regulators of microtubule dynamics, and cohesin to spindle stability has been previously studied. In this study, we examine the contribution of chromatin structure on kinetochore positioning and spindle length control. Following nucleosome depletion, by either histone H3 or H4 repression, spindle organization was examined using live cell fluorescence microscopy.

Histone repression led to a 2-fold increase in sister centromere separation and an equal increase in metaphase spindle length. Histone H3 repression does not impair kinetochores, while H4 repression disrupts proper kinetochore function. Deletion of outward force generators, kinesins Cin8p and Kip1p, shortens the long spindles observed in histone-repressed cells. Oscillatory movements of individual sister chromatid pairs are not altered following histone repression.

The increase in spindle length upon histone repression and restoration of wild-type spindle length by the loss of plus-end directed motors suggests that during metaphase, centromere separation and spindle length are governed in part by the stretching of pericentric chromatin. Chromatin is an elastic molecule that is stretched in direct opposition to the outward force generators Cin8p and Kip1p. Thus we assign a new role to chromatin packaging as an integral biophysical component of the mitotic apparatus.

Introduction

The mitotic spindle apparatus functions to segregate the replicated genome during cell division (Scholey et al., 2003). Accurate chromosome segregation is ensured by the monitoring of sister chromatid bi-orientation prior to anaphase. Once bi-oriented, sister chromatids align along the metaphase plate and are held under tension at their kinetochores, a cue that satisfies the spindle checkpoint and allows mitosis to proceed (Lew and Burke, 2003). In *Saccharomyces cerevisiae*, metaphase alignment of chromosomes results in the formation of two distinct kinetochore clusters (Goshima and Yanagida, 2000; Pearson et al., 2004). These clusters represent the average position of sister centromeres separated by kinetochore microtubule dependent forces. It has been proposed that tension-dependent rescue and a microtubule catastrophe gradient determine kinetochore microtubule length, and kinetochore clustering has been attributed to Cin8p function (Gardner et al., 2005; Tytell and Sorger, 2006). These results highlight the importance of microtubule dynamics regulation and microtubule motors in defining kinetochore position within the spindle.

In order for the spindle to hold sister chromatids under tension, the spindle must form a stable structure. Indeed, during metaphase in most organisms, the spindle maintains a stable spindle length despite the dynamics of individual microtubules and chromosomes (Gorbsky et al., 1990; Goshima et al., 1999; Yeh et al., 1995). Interpolar microtubules from opposing spindle pole bodies form an organized array that may be cross-linked by microtubule motor proteins, and/or other microtubule associated proteins (Saunders et al., 1997; Schuyler et al., 2003; Winey et al., 1995). This arrangement contributes to the stability of the two halves of the mitotic spindle during metaphase and

provides the means by which spindle pole bodies are rapidly separated from each other during anaphase B.

Metaphase spindle stability suggests that once formed, spindles are under roughly equal and opposing forces. Deletions of either *CIN8* or *KIP1* lead to abnormally short metaphase spindles, suggesting that these plus-end directed motors generate outward spindle force (via sliding interpolar microtubules against each other) (Saunders and Hoyt, 1992). Cells lacking both Cin8p and Kip1p are inviable, but deletion of the minus-end directed motor *KAR3* suppresses this lethality, suggesting that Kar3p provides an inward force that opposes and balances the outward force generated by Cin8p and Kip1p (Saunders and Hoyt, 1992). Inward spindle force has also been attributed to the cohesin complexes that link sister chromatids prior to anaphase onset (Severin et al., 2001). Neither of these hypotheses is consistent with recent data. Spindles in *kar3* mutants alone are short, a result in contrast to the prevailing model (Page and Snyder, 1992; Sproul et al., 2005; Zeng et al., 1999). Likewise, loss of cohesin does not result in complete separation of sister chromatids (Antoniacci and Skibbens, 2006).

An alternative model, based upon the physical properties of chromatin, is that chromosomes themselves behave as mechanical springs that resist outward spindle forces (Figure 2.1A). As pericentric chromatin is stretched, its resistive force increases until it is balanced with the pulling forces of the spindle. This force balance defines the separation of bi-oriented sister chromatid centromeres along the spindle, and spindle length. This model predicts that changes in chromatin structure would result in changes in kinetochore separation and spindle length.

To test this model, we have lowered chromatin packaging through the repression of histone proteins H3 and H4. Repression of histone H4 results in the decrease of nucleosome concentration within chromatin by approximately 2-fold (Kim et al., 1988). The effect of histone H3 repression was also examined because centromeric nucleosomes contain a histone H3 variant, Cse4p (CENP-A) (Meluh et al., 1998). Repression of H3 would therefore affect nucleosomes throughout the genome except at the centromeric nucleosome where it is replaced by Cse4p, while H4 repression would affect nucleosomes at all loci, including the centromere.

Our results demonstrate that kinetochore cluster separation and spindle length are both dependent on proper chromatin packaging. Histone repression leads to both increased kinetochore separation and increased spindle length. By combining histone repression with *CIN8* and *KIP1* deletions, we find that chromatin stretching is proportional to applied force. Finally, we show that nucleosome depletion results primarily in a change in chromatin's rest length. Together, these results are consistent with the model that chromatin plays a significant role as a structural element within the mitotic spindle by opposing outward, microtubule-based spindle forces.

Material and Methods

Yeast Strains and growth

Unless otherwise noted, all strains used in this study were constructed in the YEF473A background (Bi and Pringle, 1996). Relevant genotypic information can be found in Table 2.1. Spc29p fluorescent fusion proteins were created by fusing CFP (or RFP) to the C-terminus of the protein using PCR-generated integration cassettes (Bouck and Bloom, 2005; Longtine et al., 1998). Genes of fusion proteins remained under control of their endogenous promoter. Nuf2p-GFP was created by integration of the *BstEII* digestion product of pJK67.

GAL-H3 and GAL-H4 strains were constructed by first deleting *HHT1* and *HHF1*, respectively, by integration of a PCR-generated deletion cassette (Longtine et al., 1998). Next, the endogenous promoter of the second copy of each gene (*HHT2* or *HHF2*) was replaced with a PCR-generated cassette containing the *GAL1* promoter and selectable marker (Longtine et al., 1998). Cells were plated on galactose-containing selective plates. Strains were verified by death on glucose-containing plates, large-budded arrest in liquid YPD, and PCR using oligonucleotides flanking the expected sites of integration.

CIN8 deletion was carried out by integration of a deletion cassette generated by digesting pMA1186 with *PstI* and *SalI*. *KIP1* was deleted using a PCR-generated deletion cassette as previously described (Longtine et al., 1998). *MCD1-6HA* was created using pVG270 (digested with *AgeI*), kindly provided by P. Megee.

GAL-H3 and GAL-H4 strains were maintained at 32°C in galactose-containing medium. Unless otherwise noted, histone repression was carried out as follows: cells were arrested for 3 hours in G₁ with 10 µg/ml alpha factor. Next, cells were washed into glucose-containing medium (YPD) and incubated for 3 hours prior to imaging. For early/mid-S phase arrest, cells were incubated for 3 hours in media containing 200 mM hydroxyurea. For microtubule depolymerization, nocodazole was used at 15 µg/ml (dissolved in DMSO). An equal volume of DMSO alone was used as a negative control.

Microscopy

Unless otherwise noted, images were acquired at room temperature with a Nikon E600-FN microscope using a 1.4 NA 100x objective and cooled Hamamatsu Orca II camera. Cells were mounted on nutrient-containing gelatin slabs prior to imaging. Image acquisition and quantitation were performed using Metamorph 6.1 software (Universal Imaging). All distances were measured in triplicate as pixel distances using Metamorph 6.1 software (Universal Imaging) and converted to actual distance (µm). Data were exported to Microsoft Excel for analysis and presentation. While images presented in figures are maximum intensity projections of 5 plane z-series stacks, distance measurements were made using uncompiled images.

lacO-LacI-GFP images were acquired in one plane every 2 seconds. Total observation time was approximately 10 min, yielding over 300 data points for each cell type. Oscillation amplitude was defined as the distance traveled before a change in direction.

Fluorescence recovery after photobleaching (FRAP) experiments were carried out with a Nikon TE2000-U microscope using a 1.4 NA 100x objective and cooled Hamamatsu Orca ER Camera, as previously described (Pearson et al., 2004).

DraI accessibility assay

DraI accessibility at the centromere was performed as carried out previously (Saunders et al., 1990). Briefly, nuclei were isolated from spheroplasted yeast under native conditions and digested with increasing amounts of *DraI* enzyme. DNA was extracted, resolved on a 1% agarose gel, and transferred to nitrocellulose membrane. Hybridization of radiolabelled probe was detected using a phosphorimager screen, and quantitated using ImageQuant (Molecular Devices).

Chromatin immunoprecipitation

Chromatin immunoprecipitation experiments were carried out on histone repressed and non-repressed cells as previously described (Braunstein et al., 1993; Dedon et al., 1991; Weber et al., 2004). Sequences for loci amplified by PCR (at *CEN3* and the arm of ChrX) are available upon request.

Results

Pericentric chromatin constrains separated sister centromeres in metaphase

To test the hypothesis that pericentric chromatin restrains centromere separation, we lowered chromatin packaging through the repression of histone H3 or H4 and measured kinetochore cluster separation in the mitotic spindle. Two possible outcomes were predicted to follow histone repression (Figure 2.1B). If chromatin stretching does not resist the pulling forces from microtubules, then there would be no change in centromere separation following histone repression. Alternatively, if pericentric chromatin stretching is important in resisting pulling forces generated by depolymerizing kinetochore microtubules, then sister centromeres should be pulled further apart following histone repression.

To distinguish between these two outcomes, we examined kinetochore cluster separation in cells expressing Nuf2p-GFP (a kinetochore component) in which histone H3 or H4 levels could be controlled. One copy of the histone gene was deleted and the promoter of the second copy was replaced by the regulatable *GALI* promoter (see Material and Methods). Cells were grown in YPG (histone transcription on), arrested in G₁ with the mating pheromone alpha factor, and then released from this arrest into YPD (histone transcription off) for 3 h. Repression of histones resulted in cell cycle arrest with large budded cells (Kim et al., 1988). Following H3 repression, cells contained two clusters of Nuf2p-GFP, indicative of centromere separation observed in metaphase cells (Figure 2.1C). In wild-type cells, centromere clusters were separated by 0.84 μm (SD=0.23, n=71). The distance between centromeres increased to 1.60 μm in H3-

repressed cells (SD=0.34 μm , n=77). Fluorescence Recovery After Photobleaching (FRAP) analysis of bi-oriented Nuf2-GFP clusters in H3-repressed cells showed that Nuf2p-GFP remained stable, like wild-type cells (Joglekar et al., 2006). The stability of kinetochore attachments suggests that the increase in centromere separation is not a consequence of altered kinetochore function. Therefore, pericentric chromatin plays a physical role in determining the extent to which sister centromeres are stretched apart due to microtubule dependent pulling forces.

Unlike histone H3, histone H4 repression resulted in Nuf2p-GFP de-clustering in 55% of cells (Figure 2.2). This defect is consistent with impaired kinetochore formation or function upon loss of the centromeric nucleosome. In H4-repressed cells with only two kinetochore clusters, Nuf2p-GFP foci were separated by 1.38 μm (SD=0.24 μm , n=60). Thus, H4 repression leads to greater separation of sister kinetochores in the fraction of cells with clustered kinetochores.

Kinetochore de-clustering after histone H4 repression

In addition to the differences in kinetochore separation and spindle length between H3 and H4-repressed cells, Nuf2-GFP formed more than two foci following H4 repression. These foci frequently co-localized with spindle pole bodies and along the spindle axis, but were occasionally off the spindle axis as well (Figure 2.2A). We found that kinetochores were de-clustered (>2 Nuf2-GFP foci formed) in 55% of cells following H4 repression, but rarely in wild-type (5% de-clustered) or H3-repressed (10% de-clustered) cells (Figure 2.2B). Kinetochore de-clustering after H4 repression suggests that kinetochore formation or function may be compromised.

Since the centromere is known to contain a specialized nucleosome containing the H3 variant Cse4p, we considered the possibility that H4 depletion might lower the efficiency of kinetochore assembly at the centromere through disruption of the centromere specific nucleosome. To test this idea, chromatin was purified from cells grown in either YPG or YPD, and digested with *DraI* endonuclease. The budding yeast centromere contains three *DraI* endonuclease sites, but these sites are protected by centromere-kinetochore binding. As previously reported, *DraI* accessibility increased following histone H4 repression, but was not increased by H3 repression (Figure 2.1C) (Saunders et al., 1990). Disruption of proper kinetochore-centromere association may explain the kinetochore de-clustering defect observed following histone H4 repression.

Centromeres replicate in early S phase, and hydroxyurea does not prevent their replication or the assembly of kinetochores at centromeres. When cells were released from HU into YPD, the frequency of Nuf2-GFP declustering was reduced approximately 5-fold to nearly wild-type (Figure 2.2B). These results demonstrate that once centromeres were replicated and centromere-kinetochore attachments were made, lowering histone levels had no direct effect on kinetochore function. The formation of greater than two kinetochore foci in H4-repressed cells is therefore indicative of defects in kinetochore-centromere binding in cells with fewer available nucleosomes.

Chromatin structure regulates spindle length

The increased distance between sister centromeres could lead to changes in spindle structure, including shorter kinetochore microtubules, and/or increased spindle length (Figure 2.3A). To differentiate between these possibilities, we imaged Spc29p-

CFP (a spindle pole body component) and Nuf2p-GFP to determine spindle length and the position of kinetochore clusters in the spindle upon histone repression. Consistent with previous studies, the average metaphase spindle length in wild-type cells was 1.47 μm (SD=0.28 μm , n=71) (Figure 2.3B). In contrast, metaphase spindles in cells with lowered H3 levels were 2.33 μm (SD=0.40 μm , n=77), and cells with lowered H4 levels had even longer spindles (mean=2.69 μm , SD=0.36 μm , n=60) (Figure 2.3B). The increase in spindle length in H3-repressed cells is equal to the increase in kinetochore separation. These results show that spindle length is directly affected by changes in chromatin structure.

Average kinetochore microtubule length (distance from spindle pole to corresponding kinetochore cluster) was nearly identical in wild-type and H3-repressed cells (0.31 vs. 0.36 μm), while the average kinetochore microtubule length in H4-repressed cells was 0.66 μm . The small difference in kinetochore microtubule length observed between wild-type and H3-repressed cells indicates that the primary effect of increased centromere separation is increased spindle length rather than shorter kinetochore microtubules (Figure 2.3C). H4-repressed cells showed increased kinetochore cluster separation as well as longer kinetochore microtubules. In both cases, histone repression resulted in greater separation of sister kinetochore clusters, demonstrating that histone repression lowers chromatin packaging and allows for greater separation of sister centromeres in metaphase. Together, these results show that changes in chromatin structure affect spindle length and not spindle stability, suggesting that pericentric chromatin exerts an inward resistive force governing metaphase spindle length.

Cohesin mutants have been associated with altered spindle morphology (Severin et al., 2001). To address the possibility that cohesin loading or function was altered by the change in chromatin structure caused by histone repression, we assayed the association and function of cohesin in H3-repressed cells. Chromatin immunoprecipitation of cohesin subunits have previously shown an enrichment of cohesin near the centromere (Tanaka et al., 1999; Weber et al., 2004). We found that histone H3 repression did not affect Mcd1/Scclp-6HA association with the centromere (Figure 2.3D). To determine whether cohesin function (cohesion) was changed by histone repression, we treated H3-repressed cells with nocodazole and measured the frequency of sister re-association. In cells with collapsed spindles (indicative of microtubule depolymerization), 100% of sister chromatids re-associated (n=83; data not shown). Together, these results demonstrate that cohesin is both present and functional at sister chromatids. Thus the long spindles observed after histone repression are not likely due to perturbed cohesin association or function.

Spindle structure and function following histone repression

Histone repression could affect spindle length and sister kinetochore separation by indirectly affecting microtubule regulation. To test this possibility we assayed spindle stability in wild-type and H3-repressed cells.

Metaphase spindles maintain a fairly stable spindle length and kinetochore position due to the balance of forces on the structural elements of the spindle. Slight movements of the SPBs or kinetochores are likely due to stochastic on-loading and off-loading of microtubule motor proteins, and the dynamic nature of microtubule plus-ends.

To determine whether spindle stability in histone-repressed cells is comparable to wild-type metaphase spindles, we measured SPB separation in cells as they formed their spindles. We found that the slight changes in spindle length found in wild-type cells are also found after H3 repression (Figure 2.4A). In addition to single-cell analysis, populations of cells were monitored after histone repression. We found spindle length to be consistent among these cells (Figure 2.4B).

Kinetochores-microtubule attachments are stable once sister chromatid pairs are bi-oriented (Joglekar et al., 2006; Pearson et al., 2004). To determine whether histone repression affects kinetochores-microtubule stability, the stability of Nuf2-GFP in kinetochores clusters was determined by measuring the fluorescence recovery after photobleaching (FRAP). Following H3 repression, a single Nuf2-GFP cluster was photobleached and images were acquired every 30 s to monitor fluorescence recovery (Figure 2.4C). In nine cells considered, zero cells had fluorescence recovery greater than 10%, demonstrating that Nuf2p-GFP is not exchanging between the two kinetochores clusters or with the unbound, nucleoplasmic pool, consistent with previous reports in wild-type cells (Joglekar et al., 2006). FRAP analysis of Nuf2-GFP in H4-repressed cells yielded variable results, likely reflecting the heterogeneity in proper kinetochores function (data not shown). The lack of recovery in H3-repressed cells shows the stability of kinetochores-microtubule attachments, similar to wild-type cells.

The relative stability of spindle length, organization of kinetochores clusters along the spindle axis, and stability of kinetochores-microtubule attachments collectively indicate that the spindles and kinetochores formed after histone H3 depletion are functionally comparable to wild-type metaphase cells. By lowering chromatin

packaging, we have been able to isolate its contribution to the metaphase spindle, allowing us to combine this approach with other mutants to further probe the forces within the spindle.

Pericentric chromatin behaves as an elastic spindle component

Pericentric chromatin stretching could limit sister centromere separation as either an inelastic element constraining sister separation at a specific length, or as an elastic element that stretches proportionally to the force applied to it (Figure 2.5A). To distinguish between these possibilities, *CIN8* and *KIP1* were individually deleted from strains in which histone H3 levels were repressed. If chromatin is elastic, then centromere separation should be decreased in *cin8Δ* or *kip1Δ* cells; however, if chromatin is inelastic, centromere separation should not be affected (Figure 2.5A).

Histone H3 repression alone results in longer spindles (2.33 μm) and greater kinetochore separation (1.60 μm ; Figure 2.5C). Deletion of *CIN8* in H3 repressed cells resulted in spindles of approximately wild-type length (1.59 μm), and a reduction in kinetochore separation (1.13 μm ; Figures 2.5B,C). Likewise, deletion of *KIP1* in H3 repressed cells caused spindles to return to wild-type length (1.60 μm) and kinetochores were separated by approximately the same distance as wild-type cells (0.88 μm ; Figures 2.5B,C). While both motor deletions resulted in approximately the same spindle length, they did not have an equal effect on kinetochore separation (Table 2.2). This difference is likely due to different contributions of these two motor proteins. The decrease in kinetochore separation seen in both motor deletions supports the model that chromatin is an elastic element of the spindle that is stretched proportionally to the force applied to it.

Pericentric chromatin stretching contributes to the force balance that defines both centromere positioning and spindle length in metaphase.

Histone repression increases chromatin rest length

Individual sister chromatids can be visualized by integrating lac operator arrays in cells expressing LacI-GFP (Straight et al., 1997). We used lacO arrays positioned 1.8 kb from *CEN15* in wild-type and histone repressed cells to determine the effects of lowered nucleosome concentration on the position and movements of individual chromosomes (Figure 2.6A). On average, lacO foci were separated by 0.6 μm in wild-type metaphase cells, and 0.9 μm following histone H3 repression. This 50% increase in separation is consistent with the increased separation of kinetochore clusters seen following histone repression (Figure 2.1).

Based on the model that chromatin acts as a linearly elastic spring, lowering nucleosome concentration could alter either chromatin's rest length or spring constant (Gardner et al., 2005). For Hookean springs, the spring constant (k) relates the distance stretched (x) to the force (F) applied, as described by the equation $F = -kx$. If histone repression changes the spring constant (stiffness) of chromatin, we predicted that the amplitude of centromere proximal lacO array oscillations would be affected. To test this hypothesis, the distance between lacO foci was recorded every 2 seconds in wild-type metaphase cells and histone repressed cells (Figure 2.6B). The average distance traveled during an oscillation was calculated (see materials and methods). The average oscillation amplitude was 0.13 μm in wild-type cells, and 0.12 μm following histone H3 repression. Assuming there is no difference in the forces applied at kinetochores in these strains, the

similarity in oscillation amplitude suggests that spring constant is not severely altered by histone repression. Our data suggest that the primary effect of histone repression is an increase in chromatin rest length.

Discussion

A new role for chromatin packaging: structural spindle element

To test whether chromatin's biophysical properties would change by changing DNA-nucleosome packaging, nucleosome concentration within chromatin was lowered by allowing cells to replicate their DNA in the absence of histone gene transcription. Following histone repression, we observed an approximately 2-fold increase in the distance by which sister kinetochores were separated from each other due to the pulling forces of kinetochore microtubules. This change confirms that the mechanical properties of chromatin were altered after histone repression and that the force generated by the spindle is sufficient to stretch pericentric chromatin to greater lengths. The spindles in histone repressed cells also reached a longer steady state length in metaphase. Presumably, the spindle could have responded to the change in kinetochore cluster separation by either shortening kinetochore microtubules, or by extending the entire spindle length. The observed increase in spindle length suggests that, during metaphase, spindle length is being governed by the stretching of pericentric chromatin. Lowered chromatin packaging allowed for greater spindle extension in metaphase.

Outward spindle force has been attributed to the kinesin motor proteins Cin8p and Kip1p. While deletions of these motors lead to differences in anaphase spindle elongation rates, both have also been reported to form short spindles during metaphase (Saunders and Hoyt, 1992; Straight et al., 1998). To validate the model that spindle length is governed by the equilibrium reached between the outward forces (generated by motors associated with overlapping interpolar microtubules) and inward forces (including

the stretching of pericentric chromatin), we found that a new steady-state metaphase spindle length was achieved in cells lacking *CIN8* or *KIP1* in histone H3 repressed cells. In both motor deletions, spindles shortened by ~30% to approximately wild-type length, demonstrating that chromatin stretching was reduced. Thus, chromatin is an elastic molecule that is stretched in direct opposition to the outward force generators Cin8p and Kip1p.

A key element of this model is that the two Nuf2p-GFP foci observed in metaphase represent bi-oriented sister centromeres that are pulled apart. This idea is supported by three independent lines of research. First, the fluorescence intensity measured at each kinetochore cluster corresponds to 16 kinetochores (Joglekar et al., 2006). Second, fluorescence is not recovered after photobleaching of GFP fusions to kinetochore proteins in a single cluster, suggesting that once separated, sister centromeres stay separated (Joglekar et al., 2006; Pearson et al., 2004). Third, centromere proximal lacO arrays remain separated following bi-orientation (Goshima and Yanagida, 2000; Goshima and Yanagida, 2001; Pearson et al., 2001). This model of chromosome organization in the spindle correlates well with the findings in this study.

Chromatin elasticity

One interpretation of these results is that the mechanical properties of chromatin are similar to those of a mechanical spring. For a simple spring, force is directly proportional to the extent that it is stretched. In the case of bi-oriented sister chromatids in the metaphase spindle, the spindle exerts force on the chromosomes via the kinetochore microtubules until centromere flanking chromatin is pulled far enough to

reach a force equilibrium with the spindle (Figure 2.7A). While this balance of forces is demonstrated by the relatively stable spindle length seen in both wild-type and histone repressed metaphase spindles, individual chromosomes are known to oscillate along the spindle axis. The movements of individual chromosomes is likely caused by at least one of the following: (1) the stochastic binding and dissociation of microtubule motor proteins at the kinetochore, (2) the regulation or binding of other MAPs at the kinetochore, or (3) the inherent dynamic properties of microtubule plus-ends. Across the 32 kinetochores in the metaphase spindle, these imbalances are averaged out and together the sister chromatids oppose the pulling forces of the spindle.

Chromatin elasticity can be attributed to stretching of internucleosomal linker DNA, or intermolecular interactions at the DNA-protein and protein-protein levels. *In vitro* studies have shown that B-form DNA resembles a worm-like chain that takes little force (few pN) to achieve almost full extension. At full extension, increasing force must be applied until finally the molecule is permanently deformed (Gore et al., 2006; Smith et al., 1992). In contrast, chromatin pulling experiments *in vitro* have demonstrated that it behaves like an elastic polymer that requires 20 pN to dissociate a nucleosome (Brower-Toland et al., 2002). This is within the range of force estimated for single kinetochore microtubules (Grishchuk et al., 2005; Nicklas, 1983). Thus, chromatin elasticity can be assigned to protein-protein interactions of higher order chromatin packaging or DNA-protein interactions at the nucleosome. By repressing histones, the fraction of DNA associated with nucleosomes is decreased and the amount of linker DNA is increased (Figure 2.7B). Because of this shift from compacted to partially decompacted chromatin, chromatin is stretched to greater distances. No more than 50% of nucleosomes are

predicted to be absent after histone repression, so the chromatin retains its elastic properties due to the remaining nucleosomes.

Spindle differences in H3 and H4 repressed cells

While spindles were longer in both histone H3- and histone H4-repressed strains, we observed differences between these strains after histone repression. First, kinetochores (Nuf2p-GFP) were declustered in approximately half of H4-repressed cells, but rarely declustered following H3 repression. We attribute this difference to the difference in nucleosome composition at the centromere. Second, we found that spindles were slightly longer following H4 repression than H3 repression. This finding correlates well with our model that sister chromatid pairs are responsible for the primary inward force during metaphase. If each sister pair generates 1/16 of the total force in a wild-type cell, then the loss of kinetochore attachments would lead to less total force. The disorganization and declustering of kinetochores following H4 repression suggests there are kinetochore function defects and is consistent with previous work showing decreased kinetochore-centromere binding following histone H4 repression (Saunders et al., 1990). Poor kinetochore attachments result in less total inward force of stretched sister chromatids, and thus longer spindles.

Conclusion

In conclusion, this study has demonstrated a new role for the nucleosomal packaging of DNA, and presents a more complete model of the forces in the mitotic spindle. At the centromere, a specialized nucleosome is required to form the kinetochore

that links the kinetochore microtubules to the chromosomes. Flanking the centromere, the wrapping of DNA around nucleosomes functions to both package the DNA, as well as define the biophysical properties required to resist the tension placed on it. Ultimately, chromatin stretching reaches a force balance with the spindle that defines centromere separation and spindle length.

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Tables and Figures

Table 2.1: Plasmids and yeast strains used in this chapter.

<u>Plasmid name</u>	<u>Description</u>	<u>Source</u>
pMA1186	<i>CIN8::LEU2</i> plasmid (digested with <i>Pst</i> I and <i>Sal</i> I)	M.A. Hoyt
pJK67	<i>NUF2-GFP-URA3</i> integrating vector (digested with <i>Bst</i> EII)	P. Silver
pVG270	<i>MCD1-6HA-URA3</i> integrating vector (digested with <i>Age</i> I)	P. Megee
pDH7	<i>CFP-HIS3</i> plasmid	T. Davis

<u>Strain name</u>	<u>Description</u>	<u>Source</u>
YEF473A	<i>MATa trp1-63 leu2-1 ura3-52 his3-200 lys2-801</i>	J. Pringle
DCB 200.1	As YEF473A except <i>HHT1::TRP1 KAN-GAL1p-HHT2</i>	This study
DCB 220.1	As YEF473A except <i>HHF1::TRP1 KAN-GAL1p-HHF2</i>	This study
DCB 206.1	As YEF473A except <i>HHT1::TRP1 KAN-GAL1p-HHT2 MCD1-6HA-URA3</i>	This study
DCB 202.1	As YEF473A except <i>HHT1::TRP1 KAN-GAL1p-HHT2 ura3::NUF2-GFP-URA3</i>	This study
DCB 204.1	As YEF473A except <i>HHT1::TRP1 KAN-GAL1p-HHT2 ura3::NUF2-GFP-URA3 SPC29-RFP-HYG</i>	This study
DCB 222.1	As YEF473A except <i>HHF1::TRP1 KAN-GAL1p-HHF2 ura3::NUF2-GFP-URA3</i>	This study
DCB 224.1	As YEF473A except <i>HHF1::TRP1 KAN-GAL1p-HHF2 ura3::NUF2-GFP-URA3 SPC29-RFP-HYG</i>	This study
DCB 208.1	As YEF473A except <i>HHT1::TRP1 KAN-GAL1p-HHT2 ura3::NUF2-GFP-URA3 SPC29-CFP-HIS3 cin8::LEU2</i>	This study
DCB 209.1	As YEF473A except <i>HHT1::TRP1 KAN-GAL1p-HHT2 ura3::NUF2-GFP-URA3 SPC29-CFP-HIS3 kip1::LEU2</i>	This study
CEN15(1.8)-GFP	<i>MATa ade2 his3 trp1 ura3 leu2 can1 LacINLSGFP:HIS3 lacO::URA3</i> (1.8kb from CEN15)	M. Yanagida
CEN15(1.8)-GFP GAL-H3	<i>MATa ade2 his3 trp1 ura3 leu2 can1 LacINLSGFP:HIS3 lacO::URA3</i> (1.8kb from CEN15) <i>HHT1::TRP1 KAN-GAL1p-HHT2</i> .	This study

Table 2.2: Spindle organization in wild-type and histone repressed cells.

<u>Strain</u>	<u>n</u>	<u>Spindle length</u>		<u>Kinetochores cluster separation</u>		<u>Kinetochores microtubule length</u>	
		<u>Average</u>	<u>SD</u>	<u>Average</u>	<u>SD</u>	<u>Average</u>	<u>Std. Dev.</u>
Wild-type	71	1.47 ^a	0.28	0.84 ^a	0.23	0.31 ^a	0.07
GAL-H3	77	2.33 ^b	0.40	1.60 ^b	0.34	0.36 ^b	0.13
GAL-H3	41	1.59 ^a	0.64	1.13 ^c	0.70	0.22 ^c	0.12
<i>cin8Δ</i> *							
GAL-H3	75	1.60 ^a	0.31	0.88 ^a	0.24	0.36 ^b	0.09
<i>kip1Δ</i>							
GAL-H4*	60	2.69 ^c	0.36	1.38 ^c	0.24	0.66 ^d	0.15

Superscripts denote statistical significance. Data sets with identical superscripts are not significantly different ($p > 0.01$). *Only spindles with two kinetochores clusters were included in the analysis. Spindles with multiple (>2) Nuf2p-GFP foci or declustered Nuf2p-GFP were not considered.

Figure 2.1: Increased centromere separation following histone repression.

(A) Schematic model of the budding yeast spindle showing the separation of sister centromeres during metaphase. Sister chromatid arms are held together by cohesin complexes (yellow); centromeres, bound to kinetochores (black), are pulled apart by kinetochore microtubules. (B) Predicted outcomes for centromere separation following the lowering of nucleosome concentration: (i) no change indicates that chromatin structure does not affect centromere separation, or (ii) increased centromere separation indicates role of chromatin in determining centromere separation. (C) Nuf2p-GFP kinetochore clusters in wild-type, GAL-H3, and GAL-H4 cells following 3 h growth in repressive media (YPD). Histone repression results in ~2-fold increase in sister centromere separation. (Scale bar, 2 μ m)

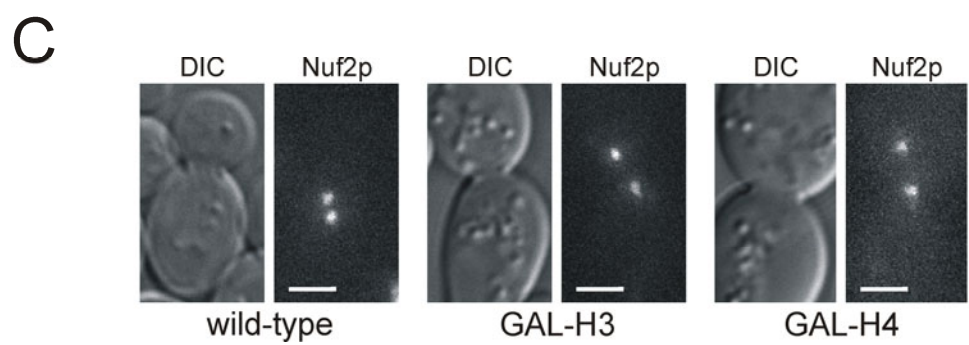
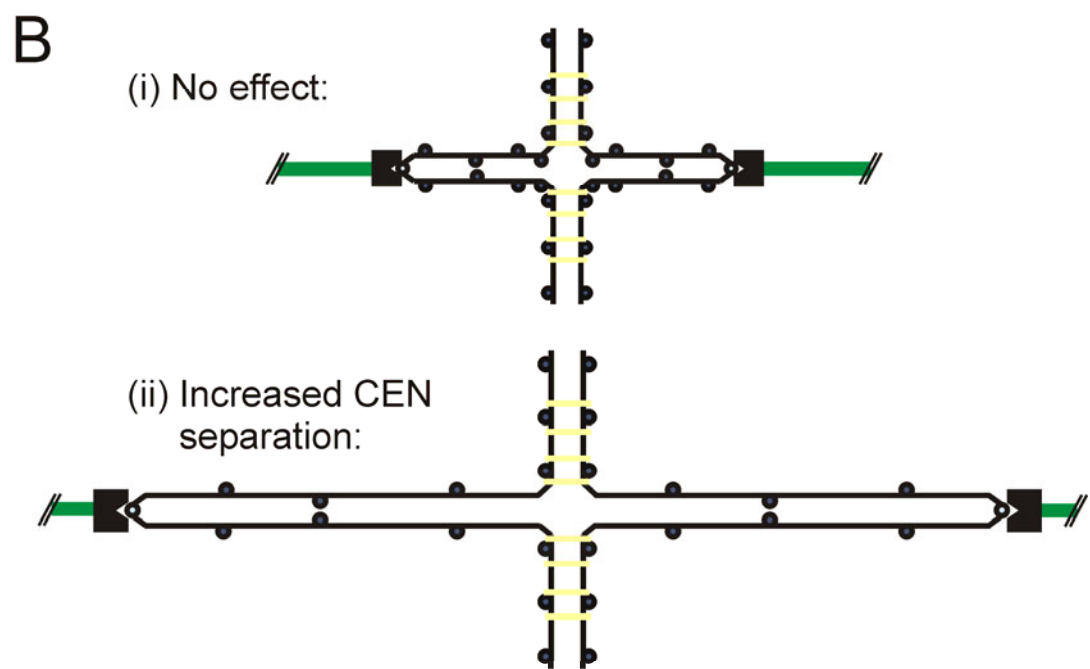
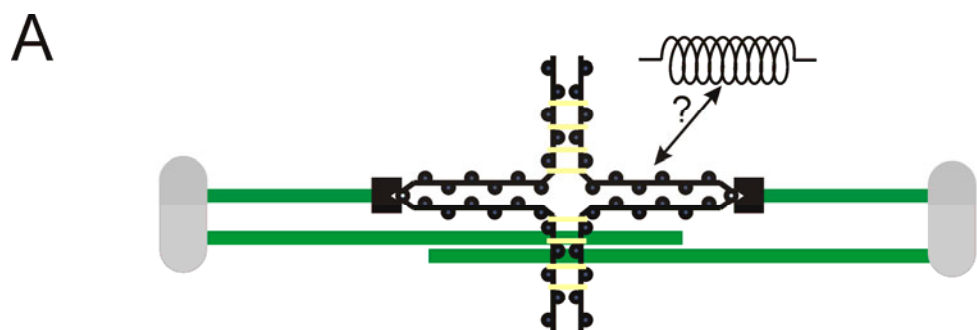


Figure 2.2: Kinetochores declustering in H4-repressed cells.

(A) Histone H4 repression leads to the formation of multiple (>2) Nuf2p-GFP foci. (B) Histone repression after release from hydroxyurea (HU) suppresses declustering frequency. (C) *DraI* accessibility at the centromere is elevated following H4 repression, but not H3 repression. (Scale bar, 2 μ m).

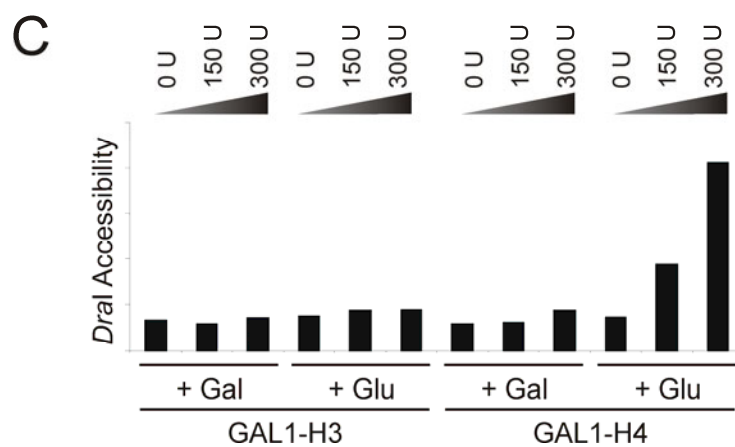
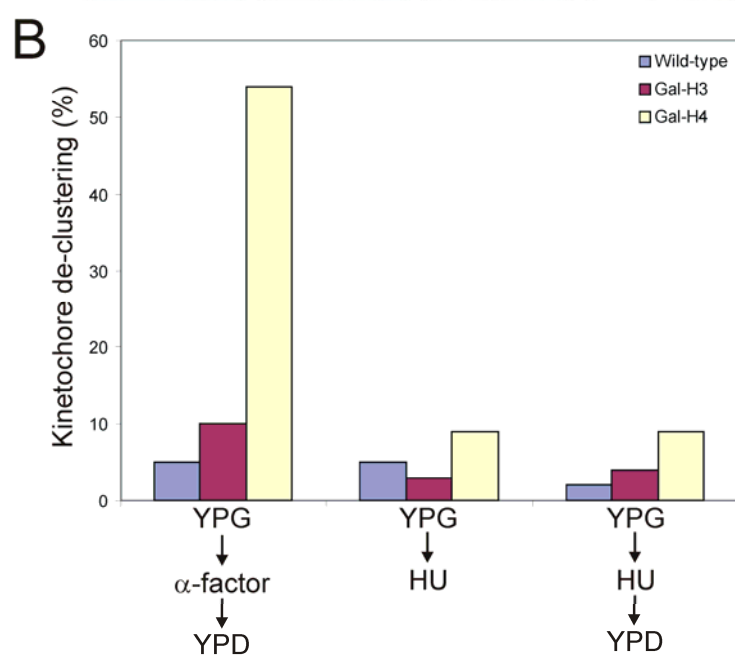
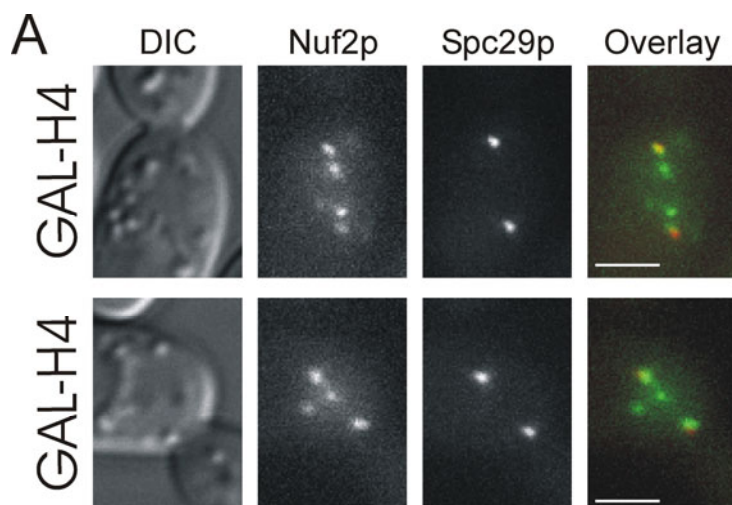
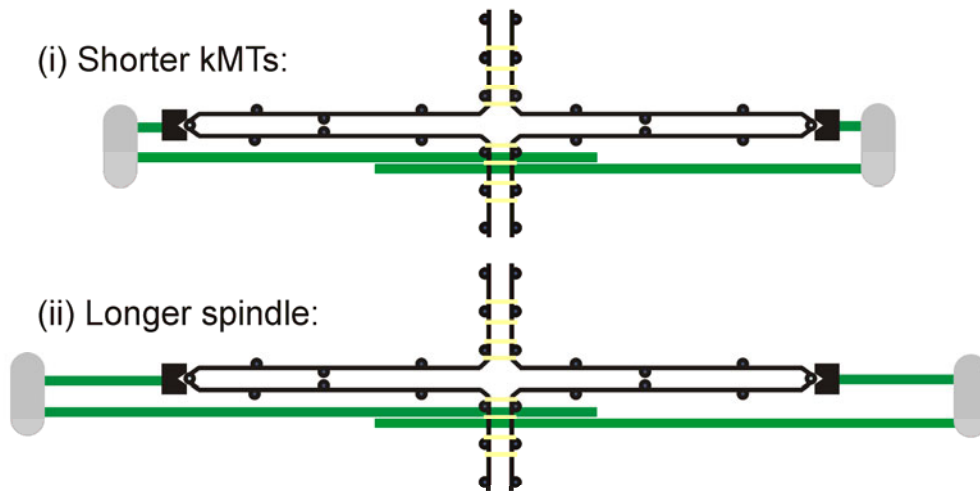


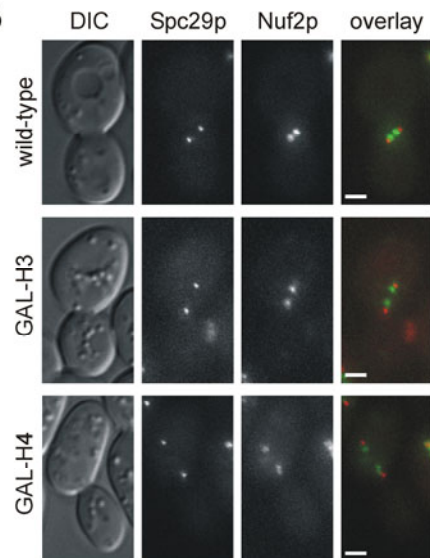
Figure 2.3: Spindle length increases following histone repression.

(A) Predicted effects of increased centromere separation on spindle structure: (i) kinetochore microtubules shorten (no change in spindle length), or (ii) the entire spindle length increases with no change in kinetochore microtubule length. (B) Spc29p-CFP (spindle pole bodies) and Nuf2p-GFP in wild-type, H3-repressed, and H4-repressed cells. (Scale bar, 2 μ m) (C) Histone repression results in increased separation of both kinetochore clusters and spindle pole bodies. Error bars represent standard deviation. (D) ChIP of Mcd1/Scc1p-6HA in GAL-H3 cells grown in permissive (YPG) or repressive (YPD) media. Centromere and arm loci were assayed for Mcd1/Scc1p association.

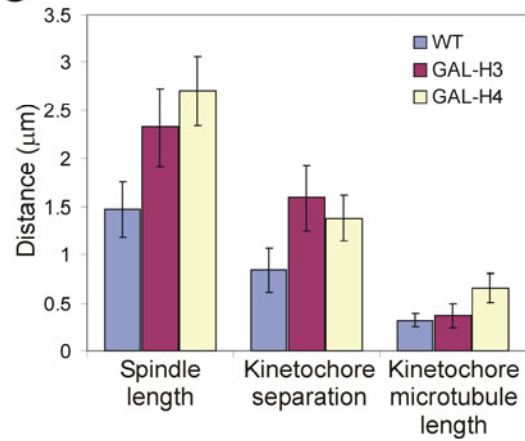
A



B



C



D

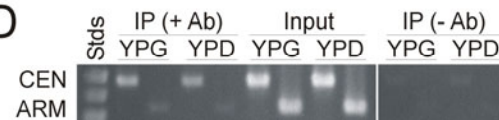
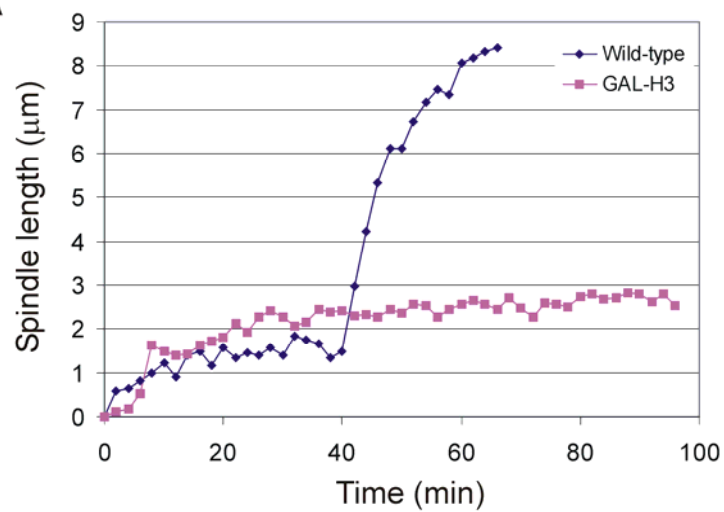


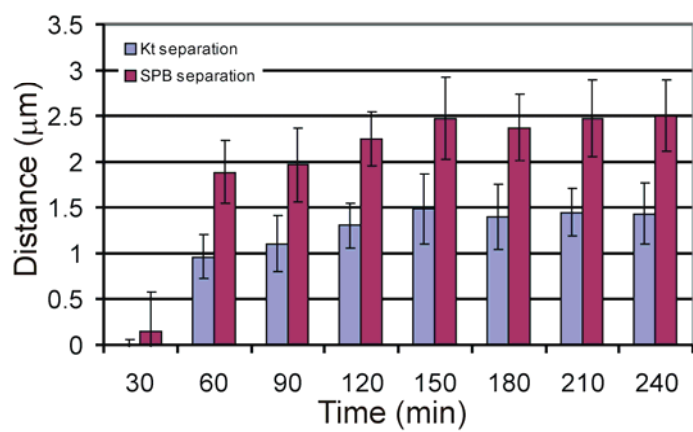
Figure 2.4: Spindle and kinetochore stability following H3 repression.

(A) Spindle length is plotted for wild-type and H3-repressed cells during spindle formation and metaphase. Wild-type cells proceed through anaphase (anaphase onset at timepoint ~40 min), while H3-repressed cells maintain a stable spindle length. (B) Kinetochore separation and spindle length in cells following release from alpha factor (T=0) under histone repression. (C) FRAP of Nuf2p-GFP in H3-repressed cells shows that Nuf2p-GFP is stable.

A



B



C

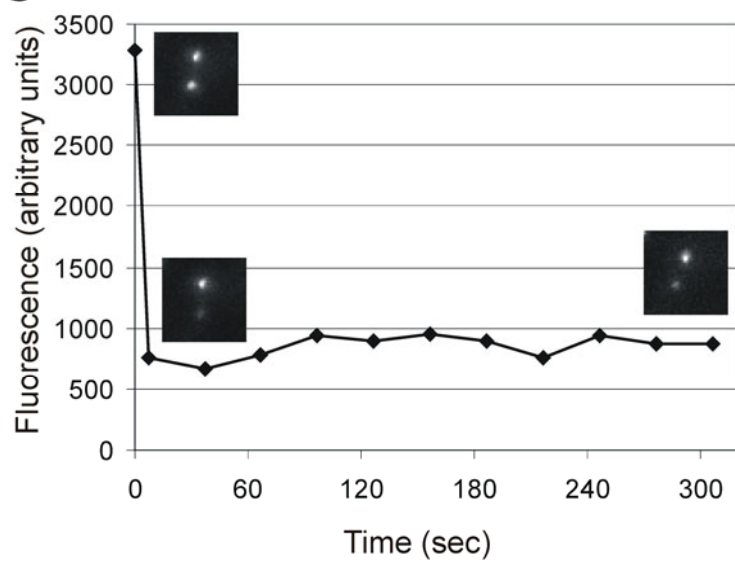
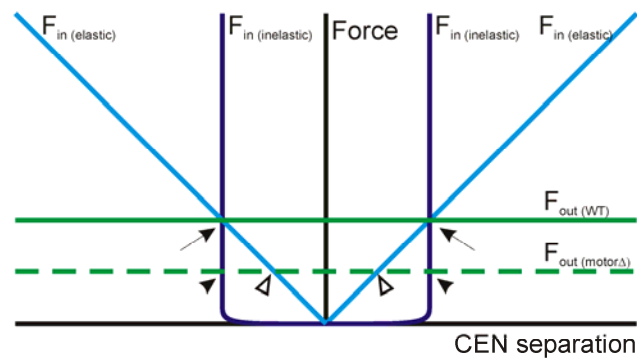


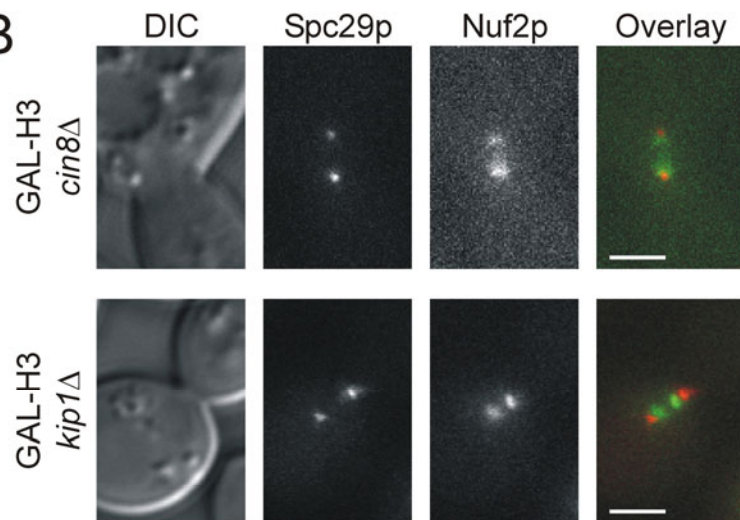
Figure 2.5: Pericentric chromatin is an elastic spindle component.

(A) Theoretical force diagram of forces acting on centromere separation. Outward forces (green lines) are assumed to be constant regardless of centromere separation distance. Deletion of *CIN8* or *KIP1* is predicted to lower outward forces (dashed green line). Inward force (blue lines) is assigned to chromatin. Elastic chromatin (light blue line) is modeled with increasing force as centromere separation increases. Assuming chromatin behaves as a Hookean spring, the slope of this line is the spring constant of chromatin. Inelastic chromatin (dark blue line) is modeled to contribute inward force only when approaching nearly full extension. Intersection points of outward and inward force lines predict length of sister centromere separation. Thus, if chromatin is inelastic, motor deletion would not change centromere separation (compare arrow to filled arrowhead). However, if chromatin is elastic, lowered force (by motor deletion) would result in less stretching and therefore reduced centromere separation (hollow arrowhead). (B) Spc29p-CFP and Nuf2p-GFP in H3-repressed cells with either *CIN8* or *KIP1* deleted. (Scale bar, 2 μ m) (C) Both spindle length and kinetochore separation are decreased in *cin8 Δ* and *kip1 Δ* cells, demonstrating that chromatin is elastic. Error bars represent standard deviation.

A



B



C

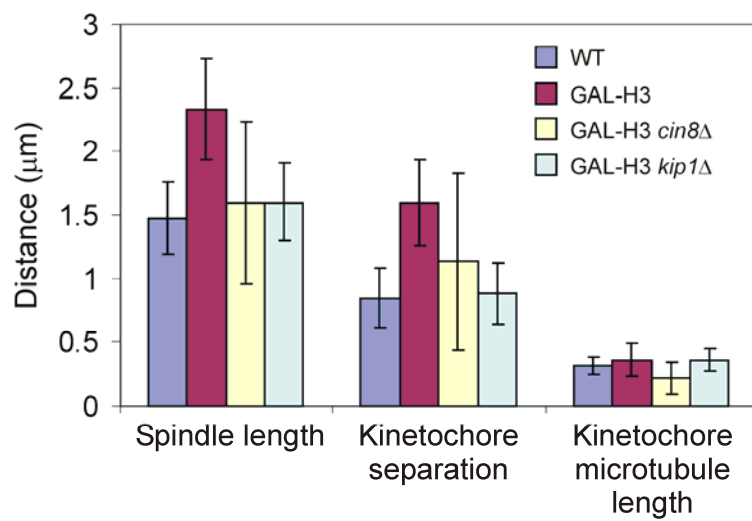


Figure 2.6: Single centromere dynamics following histone repression

(A) Kymographs of lacO arrays positioned 1.8 kb from *CEN15* in wild-type and H3-repressed cells shows dynamics of centromeres. Images were acquired in one plane every 2 sec; approximately 3 min are shown. (B) Quantitation of centromere separation and movement shows increased centromere separation following H3 repression, but similar dynamics.

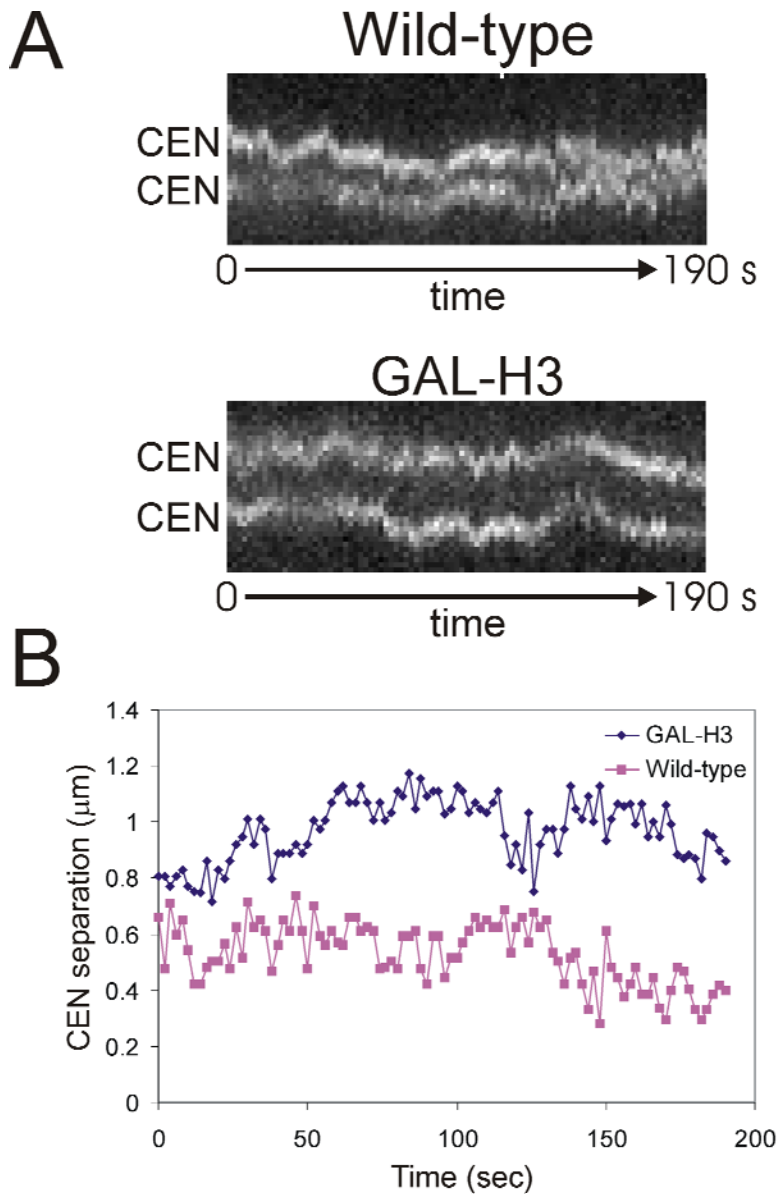
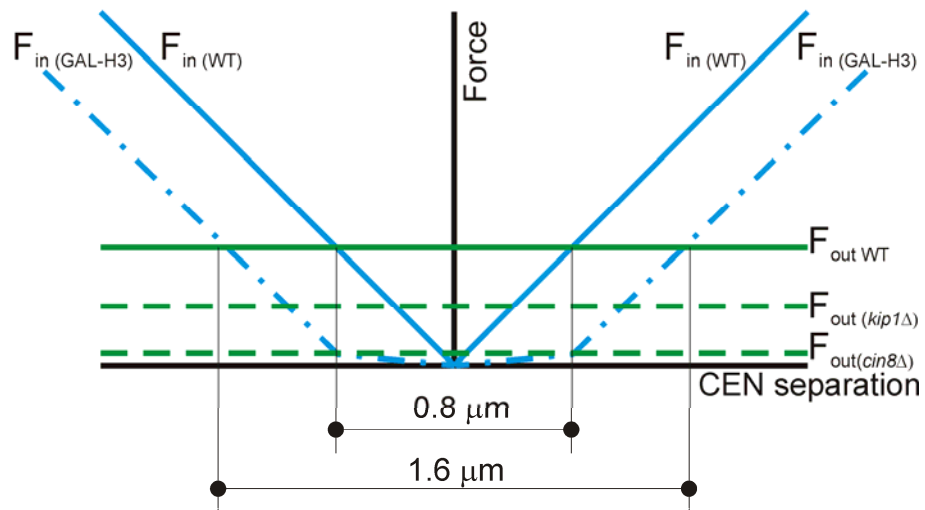


Figure 2.7: Modeling chromatin as a spring.

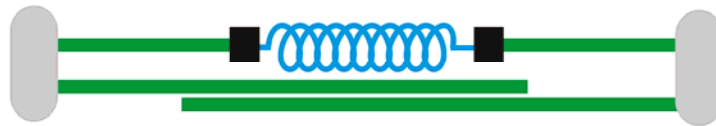
(A) Model of metaphase spindle forces based on experimental data and simplified modeling of chromatin as an elastic element. Outward spindle force is decreased in *kip1Δ* and *cin8Δ* cells and is varied by the extent that centromere separation was affected by these motor deletions (see Figure 2.5C). Inward chromatin-dependent force is shifted outward by histone repression, representing increased rest length. (B) Schematic spindle model including chromatin as a spring. Histone repression lowers the number of incorporated nucleosomes and primarily affects the chromatin spring by increasing rest length (decreasing number of “coils” in the spring), without affecting springiness (spring constant) of the remaining nucleosomes (coils). The inward, resistive force of the stretched spring contributes to balance of forces defining centromere separation and spindle length.

A

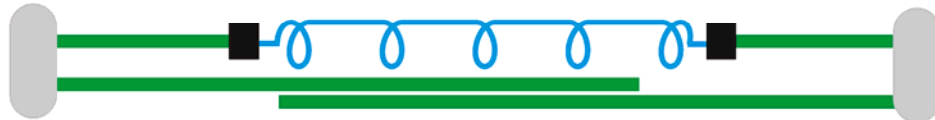


B

Wild-type:



Histone repressed:



CHAPTER THREE

THE KINETOCHORE PROTEIN Ndc10p IS REQUIRED FOR SPINDLE STABILITY AND CYTOKINESIS IN YEAST

(Contributing Authors: David Bouck and Kerry Bloom)

Abstract

The budding yeast kinetochore is comprised of greater than 60 proteins and associates with 120 bp of centromeric (CEN) DNA. Kinetochore proteins are highly dynamic and exhibit programmed cell cycle changes in localization. The CEN-specific histone, Cse4p, is one of a few stable kinetochore components and remains associated with CEN DNA throughout mitosis. In contrast, several other kinetochore proteins have been observed along interpolar microtubules and at the midzone during anaphase. The inner kinetochore protein, Ndc10p, is enriched at the spindle midzone in late anaphase. We show that Ndc10p is transported to the plus-ends of interpolar microtubules at the midzone during anaphase, a process that requires survivin (Bir1p), a member of the aurora kinase (Ipl1p) complex, and Cdc14p phosphatase. In addition, Ndc10p is required for essential, non-kinetochore processes during mitosis. Cells lacking functional Ndc10p show defects in spindle stability during anaphase and failure to split the septin ring during cytokinesis. This latter phenotype leads to a cell separation defect in *ndc10-1* cells. We propose that Ndc10p plays a direct role in maintaining spindle stability during anaphase and coordinates the completion of cell division following chromosome segregation.

Introduction

Mitosis is the process of segregating equal complements of the replicated genome to daughter cells prior to cytokinesis. Successful chromosome segregation requires the temporal regulation of sister chromatid biorientation, anaphase onset, spindle disassembly, and cytokinesis. Chromosomes are tethered to microtubules of the mitotic spindle by multi-protein, centromere (CEN)-associated complexes known as kinetochores. The kinetochore functions to capture microtubules, maintain attachment to growing and shortening microtubule plus-ends under tension, provide a framework for the spindle assembly checkpoint, and promote microtubule plus-end depolymerization at anaphase onset. Upon anaphase onset, chromosomal passenger proteins relocate from the CEN to the spindle midzone, where they are proposed to play roles in spindle stability, timing of spindle disassembly, and cytokinesis (Adams et al., 2001; Buvelot et al., 2003).

Despite its relatively simple CEN, the budding yeast kinetochore is comprised of at least 60 proteins which assemble into discrete subcomplexes (McAinsh et al., 2003). A functional kinetochore minimally requires a CEN-specific binding complex, a microtubule binding complex, and regulatory/checkpoint complexes which ensure the accuracy of attachments (Rieder and Salmon, 1998). Biochemical and genetic analyses of the kinetochore have shown that the CBF3 complex, comprised of Ndc10p, Cep3p, Ctf13p, and Skp1p, specifically binds CEN DNA (Connelly and Hieter, 1996; Lechner and Carbon, 1991). The Dam1 complex is proposed to associate directly with microtubules (Cheeseman et al., 2001; Li et al., 2002; Miranda et al., 2005b; Westermann et al., 2005). Regulation of kinetochore attachment to microtubules is likely mediated by

the Ipl1p-Bir1p-Sli15p complex (IBS) under surveillance of the spindle assembly checkpoint (Biggins et al., 1999; Cheeseman et al., 2002; Lew and Burke, 2003; Tanaka et al., 2002). The functions of other kinetochore subcomplexes and their individual proteins remain to be understood.

The labeling of kinetochore proteins with Green Fluorescent Protein (GFP) has enabled the characterization of kinetochore-CEN complexes in living cells. In G1, unreplicated kinetochores remain closely tethered to the spindle pole body (SPB) (Jin et al., 2000). Coincident with, or shortly after DNA replication, kinetochores establish attachments to one of the duplicated SPBs. Chromatids of the same replicative age are randomized between SPBs, indicating that old kinetochore-pole attachments are labile and new attachments are promoted to both old and new poles (Neff and Burke, 1991). It has been proposed that the aurora kinase Ipl1p promotes kinetochore-microtubule detachment (Tanaka et al., 2002). In this way, errors in attachment are continually corrected through detachment/attachment cycles until tension between sister chromatids is attained.

In G2/M, the sister chromatids have bioriented and kinetochore complexes appear as two clusters along the spindle axis (Goshima and Yanagida, 2000). Fluorescent labeling of individual chromosomes near the CEN has revealed that individual kinetochore-CEN complexes oscillate along the spindle until anaphase onset (Goshima and Yanagida, 2000; He et al., 2000; Pearson et al., 2001; Straight et al., 1997; Tanaka et al., 2000). Anaphase includes the movement of kinetochores to their respective SPBs in anaphase A and the elongation of polymerizing interpolar spindle microtubules (anaphase

B) until the spindle extends across the length of the budded cell (Pearson et al., 2001).

The anaphase spindle remains intact until the genomes are completely segregated.

During early anaphase, Cdc14p is activated and freed from the nucleolus. Cdc14p phosphatase activity leads to the dephosphorylation of the yeast INCENP Sli15p, resulting in the localization of Sli15, Ipl1p, and the kinetochore protein Slk19p to the midzone (Pereira and Schiebel, 2003). The budding yeast midzone consists of overlapping, anti-parallel microtubule plus-ends (Maddox et al., 2000; Winey et al., 1995). Slk19p and Sli15p have been proposed to contribute to anaphase spindle stability, while Ipl1p has been proposed to regulate the timing of spindle disassembly (Buvelot et al., 2003; Pereira and Schiebel, 2003; Zeng et al., 1999).

Completion of the budding yeast cell cycle is marked by cytokinesis and the separation of daughter cells (Wolfe and Gould, 2005). These processes are initiated by the organization of septins at the bud neck, where they form a ring. The septin ring splits into two rings prior to constriction of the actomyosin ring and forms compartments which retain cortical factors necessary for neck constriction, membrane addition, and cell wall synthesis (DeMarini et al., 1997; Dobbelaere and Barral, 2004). Division of the cytoplasm is followed by the physical separation of the adjoining cells through septum abscission.

In this work, we have used high-resolution time-lapse microscopy to examine the dynamic localization of members of the major kinetochore sub-complexes throughout the cell cycle and characterized their non-CEN localization. Kinetochore proteins follow four distinct localization patterns during anaphase spindle elongation. We have focused our study on the inner kinetochore protein Ndc10p, a member of the CBF3 complex which

localizes to both CEN DNA and the spindle midzone during anaphase (Muller-Reichert et al., 2003; Zeng et al., 1999). Ndc10p associates with the plus-ends of interpolar microtubules during spindle disassembly and continues to associate with non-kinetochore microtubules during telophase. This CEN DNA-independent association of Ndc10p with microtubules requires Cdc14p, and the budding yeast survivin homolog Bir1p. We have also examined novel *ndc10* phenotypes and found that *ndc10-1* cells exhibit spindle stability defects during anaphase spindle elongation. Additionally, *ndc10-1* cells fail to properly organize septins at the bud neck, and show defects in the last step of the budding yeast cell cycle, cell separation.

Material and Methods

Yeast Strains and Media

Plasmids used in this study are listed in Table 3.1. All strains used are listed in Table 3.2 and were constructed in the YEF473A background unless otherwise noted (Bi and Pringle, 1996). GFP fusions were made by PCR amplification of a *GFP-HIS3* cassette (from pFA6a-GFP(S65T)HIS3MX6) flanked with 50 bp of homology to the site of integration, as previously described (Longtine et al., 1998). All GFP fusions were targeted to the 3' end of the gene, unless otherwise noted. pCFPTUB1, pJK145, and pKK2 were linearized with *Stu*I, *Cla*I, and *Hind*III, respectively to produce linear DNA fragments for integration. Integration was confirmed by fluorescence microscopy.

KBY2341 was created by first integrating the linearized pKK2 fragment at *ura3*-52. Positive transformants (*URA*⁺) were then plated on 5-FOA containing plates to select for mutations in *URA3*. *ura3* isolates were then transformed with linearized pCFPTUB1 and selected for on SD –URA plates. The same strategy was used to make DCB150 from DCB141.

The *bir1*^{td} allele was created as previously described (Pearson et al., 2003). A DNA cassette was generated by PCR amplification of *KAN:pCUP:Arg:DHFR:3HA* with flanking regions of homology to the start of *BIR1*. Integration was confirmed by PCR screening. Strains with this allele were maintained at 24°C and grown in rich media supplemented with 100 µM CuSO₄. Depletion of Bir1p was carried out by growth at 37°C, without extra CuSO₄, for at least 90 min.

Cells were grown in rich or selective media, as appropriate. Azide treatment was carried out as previously described (Pearson et al., 2003). G1 arrest was induced with 15 $\mu\text{g/ml}$ α -factor.

Image Acquisition and Presentation

Images were acquired on a Nikon E600FN microscope using a 100x 1.4 NA objective. Further details of techniques and equipment used in image acquisition have been discussed elsewhere (Shaw et al., 1997). Fluorescence images were acquired as a single plane image (Figures 3.3, 3.6) or a 5-step Z-series of 0.5 μm increments (all others). Exposure times were 200-500 ms and used 2 x 2 binning. Single planes images were taken at 5-10 s intervals, while z-series images were taken at 15-60 s intervals.

All images were taken of live cells on nutrient containing gelatin slabs except for those required for Figures 3.7A, B, and 3.4, which were first fixed in 3.7% formaldehyde.

Z-series stacks were compiled by maximum projection for presentation (Shaw et al., 1997). Kymographs were created by drawing an 8-10 pixel wide line across the spindle as previously described (Maddox et al., 2000).

Z-series compilations, kymographs, distance and fluorescence intensity measurements were carried out with Metamorph software (Universal Imaging, Downingtown, PA). When necessary, data were exported to Microsoft Excel 2000 for calculations and graphing (Microsoft, Redmond, WA). Images were arranged with CorelDRAW 10 (Corel, Ottawa, Ontario, Canada).

*Examination of *ndc10-1* phenotypes*

To examine spindle defects, wild-type and *ndc10-1* cells expressing GFP-Tub1p were grown to log phase at 24°C and then treated for 3 hr with α -factor (15 μ g/ml) to synchronize cells in G1. Cells were shifted to 37°C for an additional 30 min prior to release. Cells were washed 3 times to remove α -factor and grown at 37°C. Samples were collected at 20 min intervals and fixed for 30 min in 3.7% formaldehyde at room temperature. Spindles were measured and categorized according to their length and morphology. Cells that had not yet formed bipolar spindles were classified as “monopolar.” Bipolar spindles < 3 μ m were classified as “short” spindles, 3-6 μ m bipolar spindles as “medium,” and > 6 μ m spindles as “long.” Broken/disassembled spindles were defined as those which had one SPB in both the mother and bud, but were no longer linked to each other by visible interpolar microtubules.

To examine cytokinetic/cell separation defects, wild-type and *ndc10-1* cells were grown to log phase at 24°C and then treated for 3 hr with α -factor (15 μ g/ml). Cells were shifted to 37°C for an additional 30 min prior to being washed 3 times to release from arrest. *GAL-UB-NUF2 mad2 Δ* cells were arrested in galactose medium using α -factor. After 3hr, glucose was added to 2%, and cells were incubated for 1 hr. Cells were then washed three times and released into glucose medium. After 3-4 hr from release, cells were briefly sonicated prior to imaging. Mother cells with more than one daughter bud attached were scored as multi-budded.

Lytic digestion of *ndc10-1* cells was adapted from previous work (Bi et al., 1998; Pringle and Mor, 1975). Cells were fixed for 60 min at room temperature in 3.7% formaldehyde, and then washed into 1M sorbitol. Lyticase was added to a final concentration of 80 U/ml, and cells were incubated at 37°C for 60 min prior to imaging.

Results

Kinetochore proteins are highly dynamic and exhibit programmed cell cycle changes in localization

Cse4p, the budding yeast CENP-A homologue, and Nuf2p, an essential kinetochore protein involved in checkpoint monitoring of the kinetochore (McClelland et al., 2003; Meluh et al., 1998) form two foci that represent clusters of separated sister CENs in metaphase (Pearson et al., 2004). These foci segregate to opposite SPBs during anaphase and remain proximal to the SPBs into the next cell cycle (Figure 3.1A; data not shown). Cse4p-GFP and Nuf2p-GFP are not detectable at other structures in the cell (Figure 3.1A; data not shown). This behavior reflects CEN DNA position as demonstrated using the lacO-lacI-GFP labeling method (Goshima and Yanagida, 2000; He et al., 2000; Pearson et al., 2001; Straight et al., 1997).

A second class of kinetochore protein decorates the kinetochore and microtubules. In anaphase, Ask1p-GFP (a member of the Dam1 complex) forms two kinetochore foci, in addition to its distribution along interpolar microtubules throughout spindle elongation and disassembly (Figure 3.1A and data not shown). The association of Ask1p-GFP along microtubules is consistent with the recent finding that the Dam1 complex forms ring-like structures around microtubules *in vitro* (Miranda et al., 2005b; Westermann et al., 2005). Other members of the Dam1 complex form kinetochore foci and localize along the anaphase spindle (Cheeseman et al., 2001; Li et al., 2002) (data not shown).

Among the microtubule-associated proteins, several accumulate at the spindle midzone with quantitative differences in timing and persistence. The accessory kinetochore protein Slk19p-GFP can be found in two kinetochore foci in metaphase.

Shortly after anaphase onset, Slk19p migrates to the midzone, whereupon it dissociates as anaphase elongation progresses (Figures 3.1B and 3.2A). Fluorescence intensity measurements of Slk19p-GFP at the midzone and kinetochores during anaphase progression show that Slk19p-GFP signal intensity decays more rapidly at the midzone than at the kinetochores (Figure 3.2B). By late anaphase, Slk19p-GFP is no longer detectable at the spindle midzone (Figure 3.1B).

The localization of several kinetochore components, including Ndc10p, the aurora kinase Ipl1p, and Sli15p resemble passenger proteins observed in higher eukaryotes (Cooke et al., 1987). Ndc10p forms two kinetochore foci prior to anaphase (Goh and Kilmartin, 1993). Shortly after anaphase onset (~4 min), a fraction of Ndc10p-GFP associates with the spindle, and is eventually translocated to the spindle midzone in late anaphase where it forms a cluster at the plus-ends of interpolar microtubules (Figure 3.1B). CEN-bound kinetochores remain adjacent to the SPB in late anaphase, thus the Ndc10p fraction at the midzone reflects the CEN-independent microtubule association of this protein. Treatment of cells with sodium azide inhibited the translocation of Ndc10p to the spindle midzone, suggesting that this movement is an ATP-driven process (compare Figure 3.3A with 3.3C). Upon spindle disassembly, the microtubule plus-ends depolymerize and midzone Ndc10p-GFP breaks into two foci that migrate with the shortening microtubule plus-ends to their respective poles (Figure 3.3B, asterisk). These two Ndc10p-GFP foci migrate with an average velocity of 2 $\mu\text{m}/\text{min}$ to their respective SPBs. Thus, Ndc10p accumulates at the spindle midzone as anaphase progresses and associates with microtubule plus-ends.

Ndc10p-GFP localizes to growing and shrinking microtubules

During spindle disassembly in telophase and G1 of the next cell cycle, we observed Ndc10p-GFP along projections extending from the SPBs. These projections were dynamic (making poleward and anti-poleward movements), suggesting that Ndc10p-GFP is localizing along growing and shrinking nuclear microtubules in telophase (Figure 3.4). The rates of growth and shortening were 2.4 and 1.1 $\mu\text{m}/\text{min}$, respectively, within the range of previous estimates of cytoplasmic microtubule growth and shortening (Carminati and Stearns, 1997; Tirnauer et al., 1999).

Translocation of the inner kinetochore complex CBF3 to the midzone requires Cdc14p and survivin (Bir1p)

The CEN binding CBF3 complex, including Ndc10p exhibits DNA sequence-specificity, but no direct microtubule binding (Sorger et al., 1994). Therefore we considered that Ndc10p translocation could be dependent upon one of the midzone complexes, including IBS. Ndc10p and Bir1p were previously identified as interaction partners in a two-hybrid screen, and Ndc10p is an *in vitro* substrate of the aurora kinase, Ipl1p (Biggins et al., 1999; Yoon and Carbon, 1999). To test whether Ndc10p localization to the midzone is dependent on Bir1p, we examined the localization of Ndc10p-GFP in cells lacking Bir1p (using a temperature degon *BIR1* allele, *bir1^{td}*). In wild-type cells, Ndc10p-GFP forms foci adjacent to SPBs and along the spindle in late anaphase (Figure 3.5A). In Bir1p-depleted cells, Ndc10p-GFP failed to localize along the spindle during anaphase (Figure 3.5A). In late anaphase Ndc10p-GFP formed two kinetochore foci (located adjacent to the SPBs), but failed to localize to the spindle midzone. In contrast, Ndc10p-GFP localized along the anaphase spindle at only slightly

lower levels in *ipl1-321* cells (Figure 3.5B), suggesting that Bir1p plays a direct role in the association of Ndc10p with interpolar microtubules.

The localization of Ndc10p along the anaphase spindle and to interpolar microtubule plus-ends could be unique to Ndc10p, or indicate novel localization of the entire CBF3 complex. Ctf13p, the core of the CBF3 complex, binds dimers of Ndc10p and Cep3p to form CBF3 (Russell et al., 1999). Cep3p-GFP formed two kinetochore clusters in metaphase that migrated to opposite poles at anaphase onset (data not shown). Like Ndc10p-GFP, Cep3p-GFP localized along the anaphase spindle, enriched at the midzone, and separated into two foci which moved poleward during spindle disassembly (Figure 3.5A; data not shown). However, in *bir1^{td}* cells Cep3p-GFP failed to localize to the anaphase spindle or midzone (Figure 3.5A). These results suggest that the entire CBF3 complex localizes to interpolar microtubules during anaphase, and that the CBF3 complex associates with interpolar microtubules in a Bir1p-dependent manner.

The redistribution of CBF3 to interpolar microtubules coincides with the release of the Cdc14 phosphatase from the nucleolus and spindle microtubule stability upon anaphase onset (Higuchi and Uhlmann, 2005). In addition, the accumulation of IBS to the midzone is dependent on Cdc14p activity (Pereira and Schiebel, 2003). Ndc10p-GFP failed to localize to the anaphase spindle in *cdc14-1* cells grown at 37°C, indicating that the midzone localization of CBF3, like IBS, is dependent on Cdc14p (Figure 3.5B).

Ndc10p is required for spindle stability during anaphase

In wild-type cells the anaphase spindle elongates approximately 10µm, the full length of the budded cell prior to disassembly. To test whether *ndc10-1* cells have defects

in spindle elongation, we examined spindle length and morphology in wild-type and *ndc10-1* cells synchronized in G1 and released at 37°C (Figures 3.6A,B; see material and methods). After 60 min from release, most wild-type cells had formed bipolar spindles, and nearly half (45%) had long spindles characteristic of late anaphase. By 80 min, 72% of cells had disassembled spindles, indicative of telophase. In contrast, after 60 min from release, 11% of *ndc10-1* cells had long spindles, while most had either short (38%) or medium (39%) length spindles. At no timepoint did more than 11% of *ndc10-1* cells have long spindles. Cells lacking functional Ndc10p form pre-anaphase bipolar spindles, but these spindles fail to fully elongate during anaphase.

Time-lapse imaging of spindles in *ndc10-1* cells grown at restrictive temperature revealed cycles of partial elongation and collapse, without full elongation along the mother-bud axis (Figures 3.6C,D). These cycles of failed spindle elongation support the finding that anaphase spindle stability requires functional Ndc10p.

Spindle elongation defects in *ndc10-1* cells might be a consequence of loss of kinetochore function, rather than loss of Ndc10p function. To explore this possibility, kinetochore function was disrupted by placing the kinetochore component Nuf2p under control of the inducible *GAL1* promoter. In addition, the spindle checkpoint gene *MAD2* was deleted to allow anaphase progression in the absence of a kinetochore. This strain showed no defects in the formation of long spindles following Nuf2p depletion (Figure 3.7). Thus functional Ndc10p, and not the kinetochore, is required for the stability of elongating spindles.

ndc10-1 cells exhibit defects in cytokinesis and cell separation

Passenger proteins have been postulated to contribute to cytokinesis in tissue cells. To determine whether Ndc10p exhibits similar function at spindle microtubule plus-ends in anaphase, we examined the terminal cell morphology of *ndc10-1* cells. Cells were released from G1 arrest and grown for 3-4 hr at 24 or 37°C. While *ndc10-1* cells grown at permissive temperature showed no unusual cell morphology (0 of 100 cells were multi-budded), 83% of *ndc10-1* cells at 37°C had multiple buds attached to the same mother (n=100; Figure 3.8A). Wild-type cells grown at 37°C showed no unusual morphology (3% multi-budded; n=100). The multi-budded phenotype is indicative of multiple cell cycles in the absence of division. Thus, *ndc10-1* cells fail to complete cell division.

To explore this defect in cell division, we examined the localization of the septin Cdc3p in *ndc10-1* cells. In wild-type cells, the septin ring splits into two at the bud neck prior to constriction of the actomyosin ring (Figure 3.8B). In *ndc10-1* cells, Cdc3p-GFP localized to the neck as a single ring, but failed to split (n=60; Figure 3.8B). This defect in septin organization indicates that Ndc10p is required for a critical step at the end of the cell cycle.

Multi-budded cells could arise from defects in cytokinesis, or the physical separation of the adjoining cells through the degradation of the septum. To determine whether Ndc10p is required for cytokinesis or cell separation, *ndc10-1* cells were fixed and digested with lyticase. This treatment results in the de-clustering of multi-budded cells defective in cell separation, while cells which have failed to undergo cytokinesis remain clustered (Bi et al., 1998; Pringle and Mor, 1975). Multi-budded *myo1Δ* cell clusters, which have a defect in cell separation dissociated following digestion, while

cdc14-1 cells, which arrest in late anaphase (prior to cytokinesis) remained clustered (Figure 3.9) (Bi et al., 1998; Granot and Snyder, 1991). We found that lyticase treatment of *ndc10-1* cells released the cell clusters, indicating that *ndc10-1* cells undergo cytokinesis, but not cell separation (Figure 3.9).

The multi-budded phenotype seen in *ndc10-1* cells is similar to the morphology of spindle checkpoint mutants treated with microtubule depolymerizing agents (Hoyt et al., 1991; Li and Murray, 1991). *NDC10* has also been reported to be essential for spindle checkpoint activation (Fraschini et al., 2001). Together, these observations suggest that the multi-budding seen in *ndc10-1* cells might be an effect of the loss of kinetochore-microtubule attachment in a checkpoint deficient background. To examine the possibility that other kinetochore defects in a checkpoint deficient strain could lead to multi-budding, we examined the morphology of *GAL1-UB-NUF2 mad2Δ* cells after four hours of *NUF2* repression in glucose containing medium. Depletion of Nuf2p in a spindle checkpoint deficient strain did not result in multi-bud phenotype (data not shown), suggesting that Ndc10p, and not the kinetochore, is essential for successful cell separation.

Discussion

We have demonstrated that the major CEN DNA binding complex (CBF3) accumulates at the spindle midzone during anaphase and along growing and shrinking microtubule plus-ends in telophase and G1 (Figures 3.3,3.4). Studies of CBF3 have focused on its role in kinetochore formation at CEN DNA. The finding that CBF3 localizes to microtubule plus-ends reveals critical roles for CBF3 in stabilizing the anaphase spindle and ensuring that cytokinesis follows chromosome segregation to the spindle poles.

Ndc10p stabilizes the anaphase spindle

In the absence of functional Ndc10p, the spindle fails to fully elongate during anaphase (Figure 3.6). Spindle elongation was not compromised strains lacking kinetochores (*GAL1-UB-NUF2*) (Figure 3.7). Thus Ndc10p is specifically required for spindle stability. Ndc10p association with the anaphase spindle is dependent on the Cdc14p phosphatase that has recently been shown to dampen anaphase spindle microtubule dynamics (Figure 3.5B) (Higuchi and Uhlmann, 2005). Additionally, microtubule dynamics are regulated primarily at the plus-end in budding yeast (Maddox et al., 2000). These findings indicate that Cdc14p-dependent release of Ndc10p to microtubule plus-ends may contribute to microtubule stability in the anaphase spindle. We found that spindles in *ndc10-1* cells collapsed by the 5-6 μm stage of spindle elongation, when there are relatively few interpolar microtubules (<4 per SPB). Anaphase spindle elongation provides the motive force for chromosome segregation in yeast. It is

therefore critical that the spindle midzone remain intact during anaphase spindle elongation.

Ndc10p is required for completion of cell division

The accumulation of multi-budded cells in *ndc10-1* mutants indicates that Ndc10p is required for cell separation prior to entry into the next cell cycle (Figure 3.8A). The multi-budded phenotype is similar to *mad1,2, 3* and *bub1,2, 3* mutants treated with microtubule depolymerizing agents (Hoyt et al., 1991; Li and Murray, 1991). In the absence of the spindle checkpoint, cells attempt to re-enter the cell cycle despite their inability to maintain a mitotic spindle. Likewise, in *ndc10-1* cells, spindle checkpoint function is lost and spindle structure is compromised, suggesting the importance of both checkpoint function and spindle integrity for completion of cell division and regulated entry into the next cell cycle. However, the lack of multibudded cells upon depletion of Nuf2p indicates that Ndc10p, and not the kinetochore itself is essential for cell separation.

The failure to complete cell division in *ndc10-1* cells was confirmed by the altered organization of septins in these cells. Examination of the Cdc3p-GFP localization in *ndc10-1* cells revealed a failure of the septin ring to divide into two rings during cytokinesis (Figure 3.8B). The septins are thought to recruit and/or maintain the exocytic machinery involved in secretion at the bud neck (Dobbelaere and Barral, 2004). Failure to split the septin rings would presumably result in defects during cytokinesis. However, the terminal morphological phenotype of *ndc10-1* cells is a failure to separate cells rather than complete cytokinesis (as assayed by cell cluster dispersion following enzymatic

digestion of the cell wall). This suggests that septin defect in *ndc10-1* cells might cause a malformed septum during cytokinesis, resulting in a structure that cannot be dissolved during cell separation.

In higher eukaryotes, the spindle midzone and the delivery of passenger proteins to microtubule plus-ends appear to be essential for proper cytokinesis (Canman et al., 2003). Furthermore, the requirement of Ndc10p for cytokinesis provides a mechanism that ensures chromosome segregation to the poles prior to cytokinesis. The key regulatory step could be the release of Ndc10p to the midzone upon anaphase onset.

Non-kinetochore CBF3 forms a “pre-kinetochore”?

The localization of CBF3 to non-kinetochore microtubules is evident from telophase and persists into G1 of the next cell cycle. Ndc10p localizes to both growing and shortening microtubules emanating from the SPB (Figure 3.4). This behavior is reminiscent of microtubule “search-and-capture” mechanisms that facilitate the establishment of kinetochore-microtubule attachments in tissue cells. CEN DNA is replicated early in S phase, and sister chromatid biorientation can occur prior to the completion of DNA replication (Goshima and Yanagida, 2000; McCarroll and Fangman, 1988). Thus, early in the cell cycle, kinetochore-microtubule attachments can be made. These “pre-kinetochores” are likely to contain the IBS complex, as Bir1p is required for Ndc10p plus-end localization. IBS promotes biorientation of sister kinetochores during “search and capture” by promoting kinetochore-microtubule detachment until tension is attained (Tanaka et al., 2002). These “pre-kinetochores” could mature into functional kinetochores upon CEN DNA binding and the recruitment of the additional kinetochore

complexes which would confer stability and checkpoint function to the structure. The essential functions of kinetochores are CEN DNA binding and association with dynamic microtubule plus-ends. CBF3 was first identified as a CEN binding complex, and more recently Ndc10p has been localized along microtubules of both long and short spindles (Lechner and Carbon, 1991; Muller-Reichert et al., 2003). Our data reveal that CBF3 associates with both growing and shrinking microtubules independent of CEN DNA, and Ndc10p provides critical functions in spindle stability and cytokinesis.

Acknowledgements

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Tables and Figures

Table 3.1: Plasmids used in this chapter.

Plasmid Name	Description	Source
pCFPTUB1	<i>ura3::CFP-TUB1-URA3</i> integrating vector	M. Segal
pKK1	<i>CSE4-GFP</i> fusion, pRS314/ <i>TRP1</i>	Baker/ Fitzgerald-Hayes
pKK2	<i>ura3::NDC10-GFP-URA3</i> integrating vector	Baker/ Fitzgerald-Hayes
pDCB50	<i>ura3::CDC3-GFP-URA3</i> integrating vector	This study
pBJ1212	pFA6-KANMX-pCUP-ts Degron-3HA	Heil-Chapdelaine/Cooper
pJK145	<i>SLK19-GFP-NUF2 3' UTR URA3</i> integrating vector	Zeng <i>et al.</i> (1999)

Table 3.2: Yeast strains used in this chapter.

Strain Name	Relevant Genotype	Source
YEF473A	<i>MATa trp1-63 leu2-1 ura3-52 his3-200 lys2-801</i>	Bi and Pringle (1996)
GT1	As YEF473A except <i>ura3-52::GFP-TUB1-URA3</i>	Maddox <i>et al.</i> (2000)
KBY2310	As YEF473A except <i>NDC10-GFP-HIS3</i>	This study
DCB129	As YEF473A except <i>NDC10-GFP-HIS3</i> <i>KAN:pCUP:Arg:DHFR^{ts}:3HA:BIR1</i>	This study
DCB110	As YEF473A except <i>CEP3-GFP-HIS3</i>	This study
DCB127	As YEF473A except <i>CEP3-GFP-HIS3</i> <i>KAN:pCUP:Arg:DHFR^{ts}:3HA:BIR1</i>	This study
KBY2011	As YEF473A except <i>cse4::HYG pKK1</i>	Pearson/Bloom
KBY2090	As YEF473A except <i>slk19::SLK19-GFP-URA3</i>	Pearson/Bloom
KBY2341	As YEF473A except <i>ura3-52::NDC10-GFP-URA3* ura3::CFP-TUB1-URA3</i>	This study
DCB141	As YEF473A except <i>LEU2-GAL1-UB-NUF2 mad2::URA3</i>	This study
DCB150	As YEF473A except <i>LEU2-GAL1-UB-NUF2 mad2::URA3* ura3::GFP-TUB1-URA3</i>	This study
DCB149	As YEF473A except <i>ura3-52::CDC3-GFP-URA3</i>	This study
RNY471	As YEF473A except <i>MATα myo1::KAN</i>	J. Pringle
KBY2161	<i>MATa ndc10-1 ura3 leu2 lys2 his3 trp1 ade2 ura3-52::GFP-TUB1-URA3</i>	Pearson <i>et al.</i> (2003)
ICY211D	<i>MATα his3Δ200 leu2-3,112 ura3-52 lys2 ASK1-GFP-KAN</i>	Cheeseman/Barnes
DCB140	<i>cdc14-1 NDC10-GFP-KAN</i>	This study
SBY631	<i>ura3-1 leu2-3,112 his3-11 trp1-1 LYS2 bar1 can1-100 ade2-1 ipl1-321</i>	S. Biggins
DCB139	As SBY631 except <i>NDC10-GFP-KAN</i>	This study
DCB 148	<i>MATa ndc10-1 ura3 leu2 lys2 his3 trp1 ade2 ura3-52::CDC3-GFP-URA3</i>	This study

Figure 3.1: Anaphase localization of kinetochore proteins exhibits four distinct classes.

Selected frames from time-lapse observation of live cells expressing Cse4p-GFP, Ask1p-GFP, Slk19p-GFP, or Ndc10p-GFP. (A) Cse4p-GFP is detectable only as two bright kinetochore clusters. Ask1p-GFP remains bound along microtubules and at kinetochores. (B) Slk19p-GFP localizes to kinetochores and the midzone (arrow), but midzone signal decreases as anaphase proceeds. Ndc10p-GFP localizes along the spindle in anaphase and is enriched at the midzone (arrow) prior to spindle disassembly. Scale bar, 5 μm .

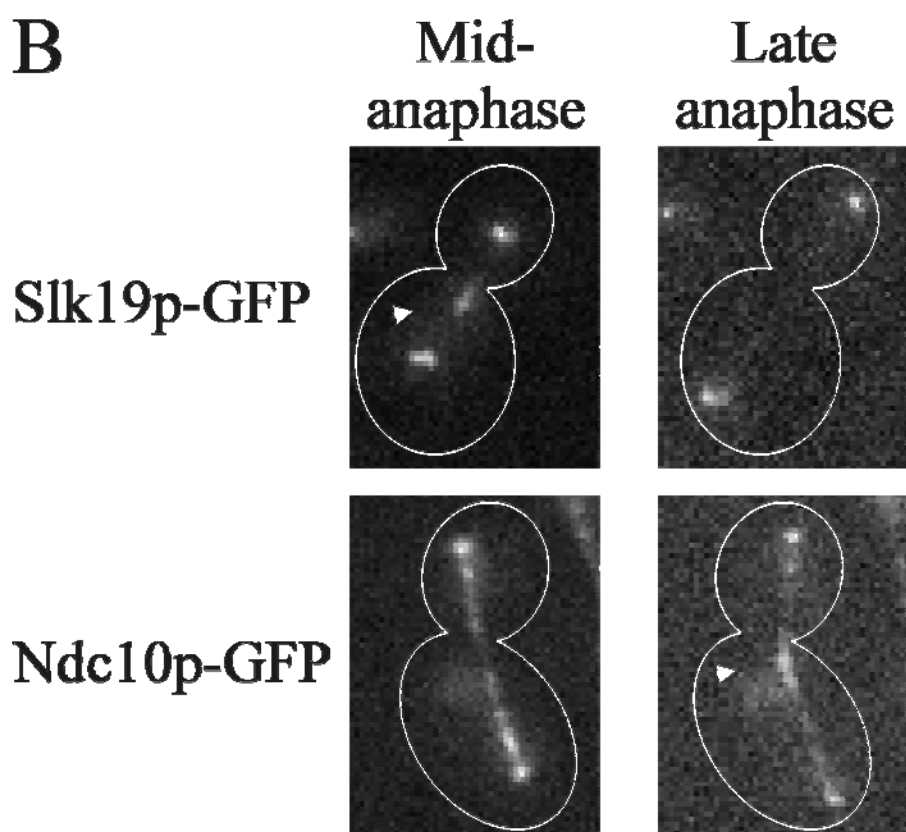
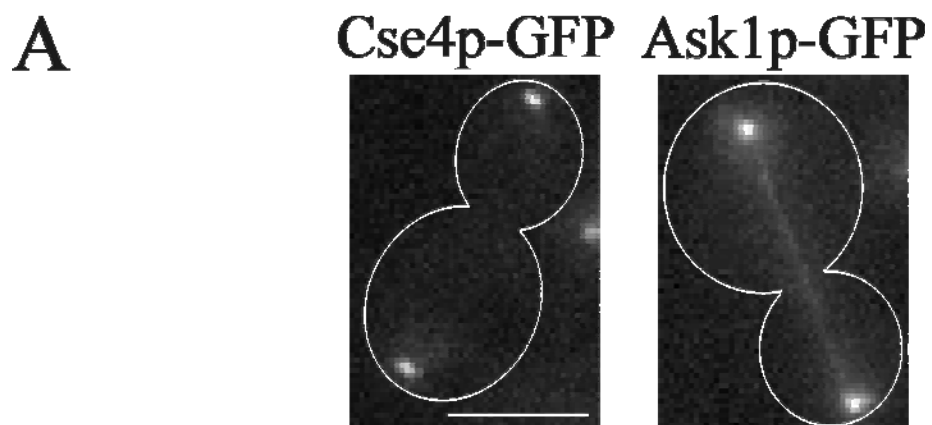


Figure 3.2: Midzone Slk19p-GFP decays as anaphase proceeds.

(A) Kymograph across the spindle of a cell expressing Slk19p-GFP. Spindle elongates from metaphase (top of kymograph) through telophase (bottom of kymograph). (B) Fluorescence intensity measurements of the same time-lapse were plotted to show that midzone Slk19p-GFP levels decrease more rapidly than at either the mother kinetochore (mKt) cluster or daughter kinetochore (dKt) cluster. Fluorescence levels of both kinetochore clusters decrease over time due to photobleaching incident to image acquisition.

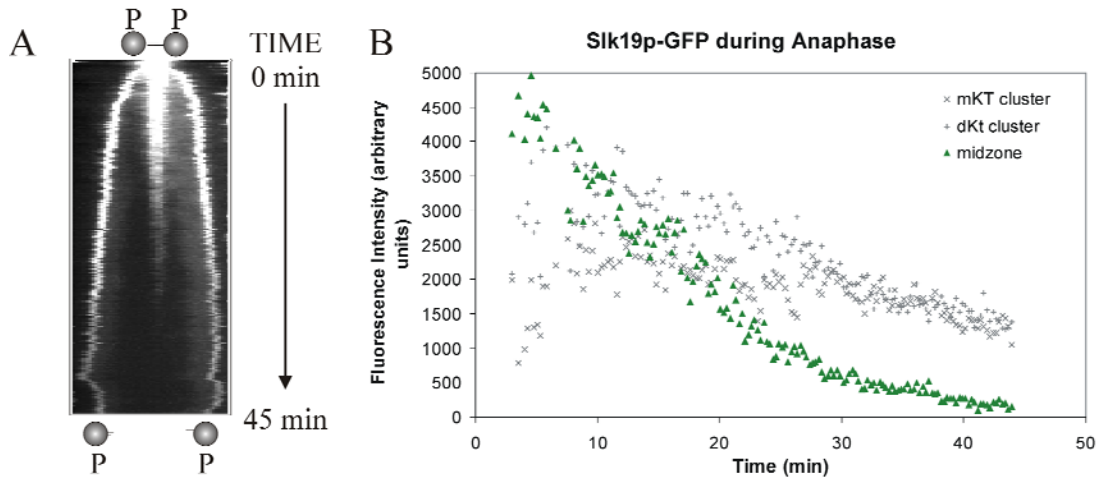


Figure 3.3: Dynamic translocation of Ndc10p-GFP to the anaphase spindle midzone.

(A) Kymograph of Ndc10p-GFP in a late anaphase spindle shows movement toward the midzone. The kymograph shows movement along the spindle through time. Vertical lines represent static foci, while movement is shown as sloped lines (arrows indicate instances of movement toward the midzone). (B) Midzone Ndc10p-GFP tracks the plus-ends of the depolymerizing interpolar microtubules during spindle disassembly (asterisk marks start of spindle disassembly). CFP-Tub1p indicates spindle disassembly is coincident with the movement of midzone Ndc10p-GFP to the SPBs. (C) Sodium azide treatment of cells inhibits the translocation of Ndc10p-GFP along anaphase spindle. Arrows indicate static Ndc10p-GFP foci along the anaphase spindle.

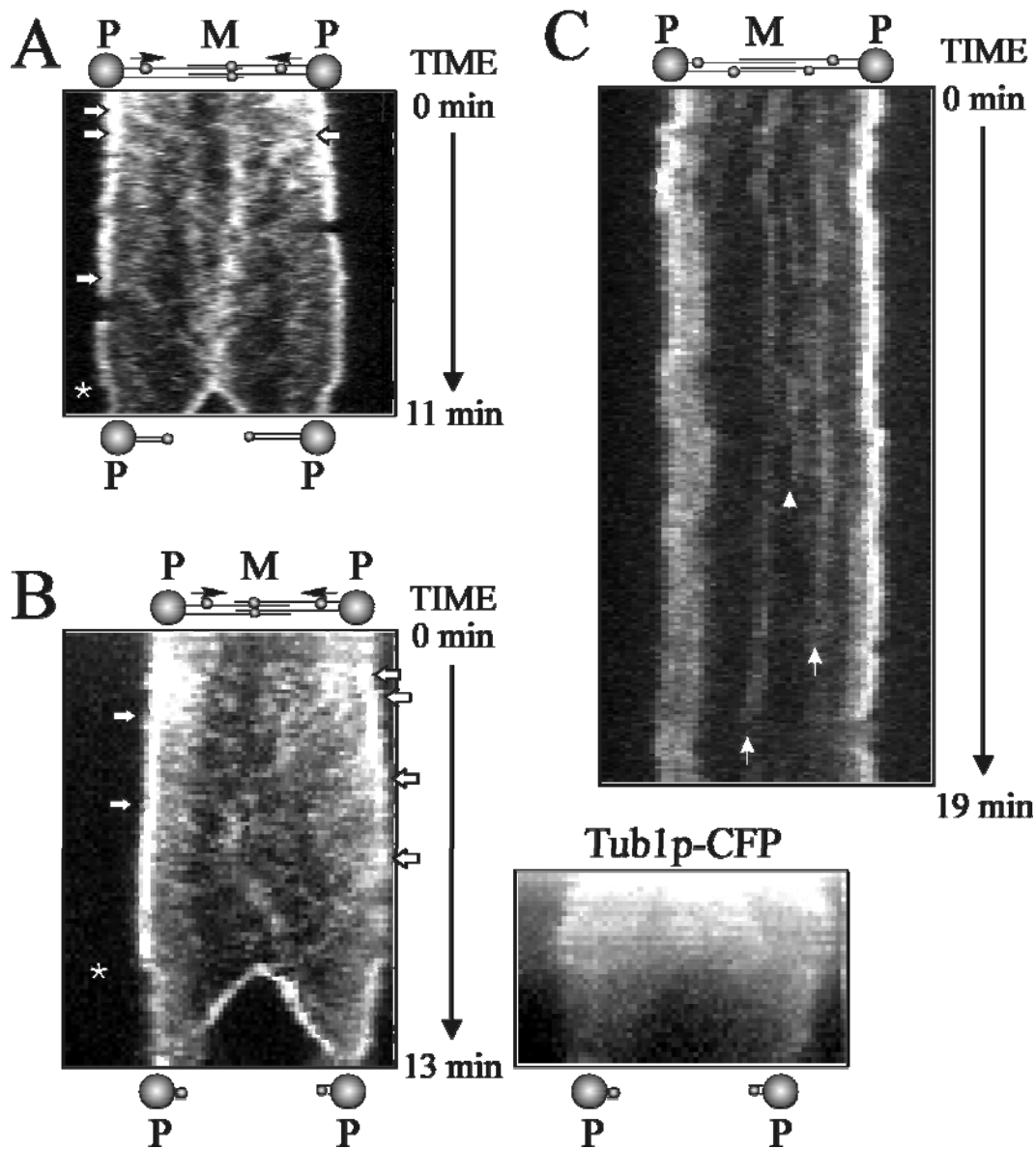


Figure 3.4: Ndc10p-GFP localizes with growing and shortening microtubules.

Time-lapse of a late anaphase cell expressing Ndc10p-GFP. At first timepoint, spindle disassembly has just begun and midzone Ndc10p-GFP has broken into two clusters (hollow arrowheads). As these clusters follow the plus-ends of depolymerizing microtubules, other projections of Ndc10p-GFP grow and shrink from the region of the SPB (white arrows). Scale bar, 5 μ m.

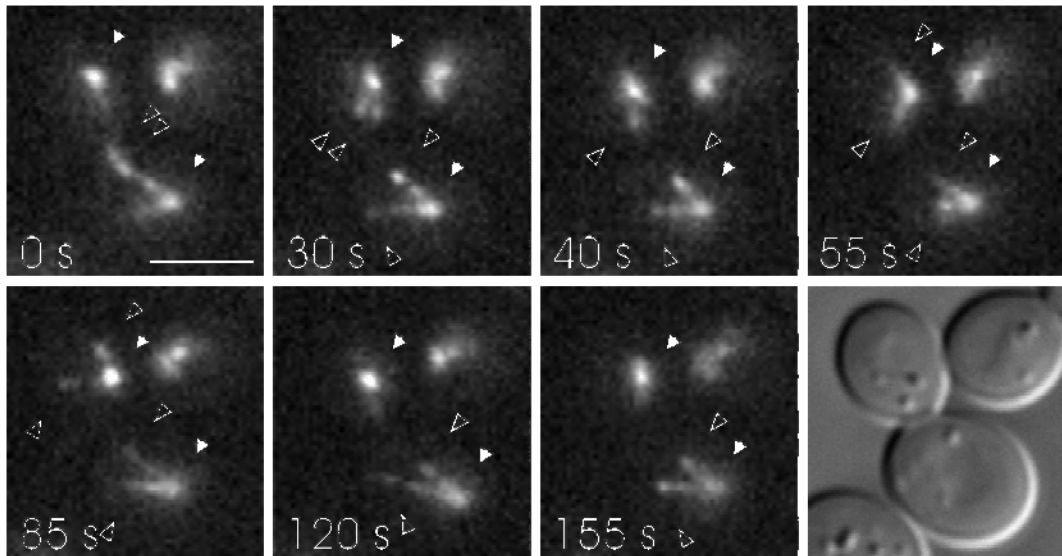


Figure 3.5: CBF3 association with anaphase spindle requires Bir1p and Cdc14p.

(A) Localization of two CBF3 components, Cep3p and Ndc10p, in wild-type cells shows localization along the anaphase spindle. Loss of Bir1p, using a temperature-degrogen allele of *BIR1* (see material and methods) results in loss of CBF3 association with interpolar microtubules and the spindle midzone. (B) Ndc10p-GFP localization in *ipl1-321* and *cdc14-1* cells grown under non-permissive conditions. Scale bar, 5 μ m.

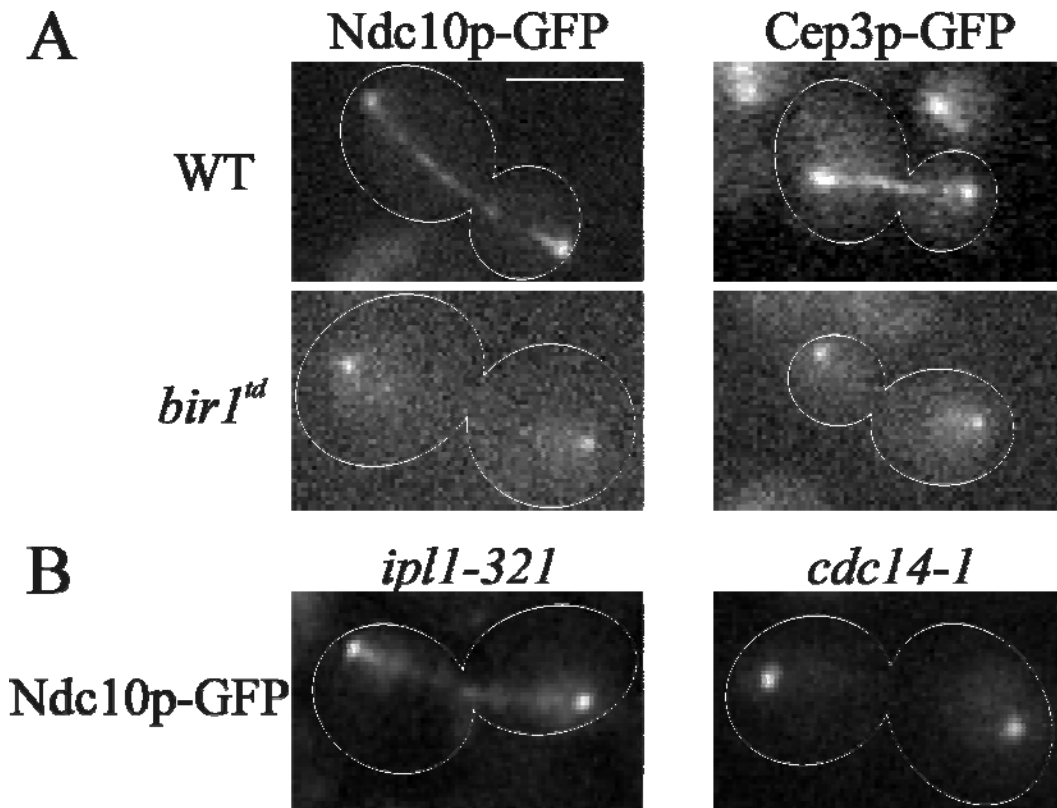


Figure 3.6: Ndc10p is required for spindle stability during anaphase and cell separation.

Wild-type and *ndc10-1* cells were arrested in G1 with α -factor and released at 37°C. Spindles were categorized as being monopolar, short, medium, long, or broken/disassembled based on morphology and length of GFP-Tub1p fluorescence (see Materials and Methods). (A) Spindle lengths of wild-type cells released from G1 arrest at 37°C. (B) Spindle lengths of *ndc10-1* cells released from G1 arrest at 37°C. (C) Time-lapse fluorescence images of GFP-Tub1p in *ndc10-1* cells grown at 37°C (bar, 5 μ m). (D) Quantitation of spindle length for cell presented in (C).

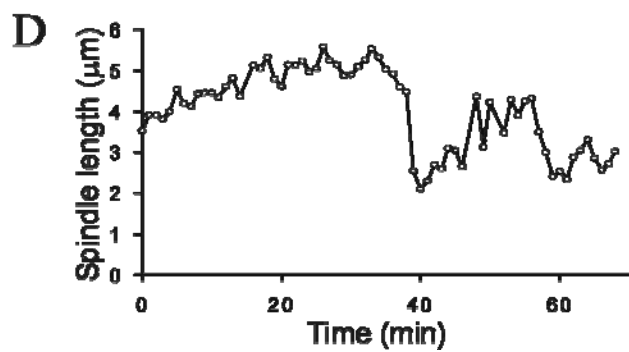
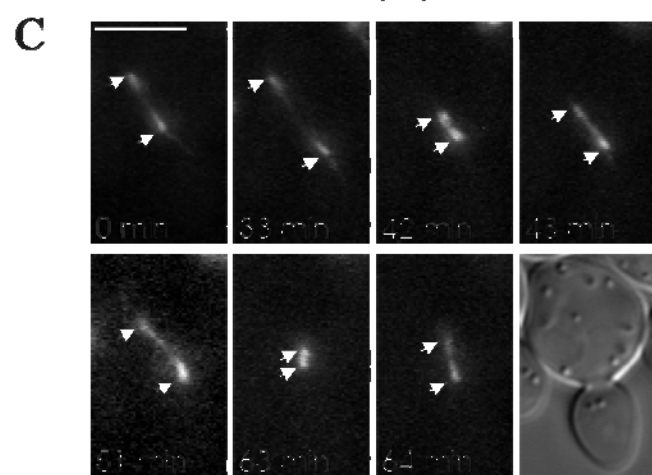
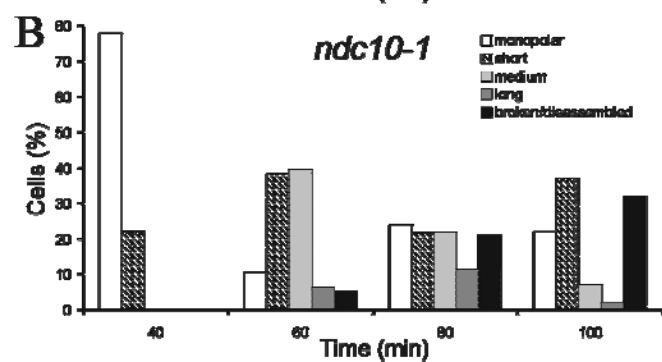
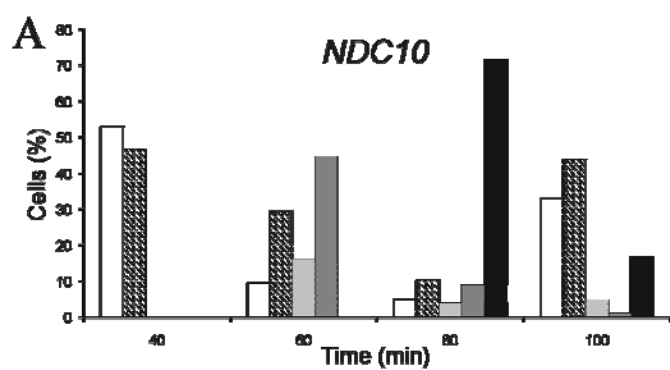


Figure 3.7: Spindle length distribution after Nuf2p depletion.

Wild-type, *GAL-NUF2*, and *GAL-NUF2 mad2Δ* cells were grown logarithmically in galactose-containing medium, washed into glucose containing medium, and imaged after 3 hr of growth. Nuf2p depletion led to an enrichment of cells with short spindles (presumably due to activation of the spindle checkpoint), while the deletion of *MAD2* allowed spindle elongation to proceed like wild-type cells.

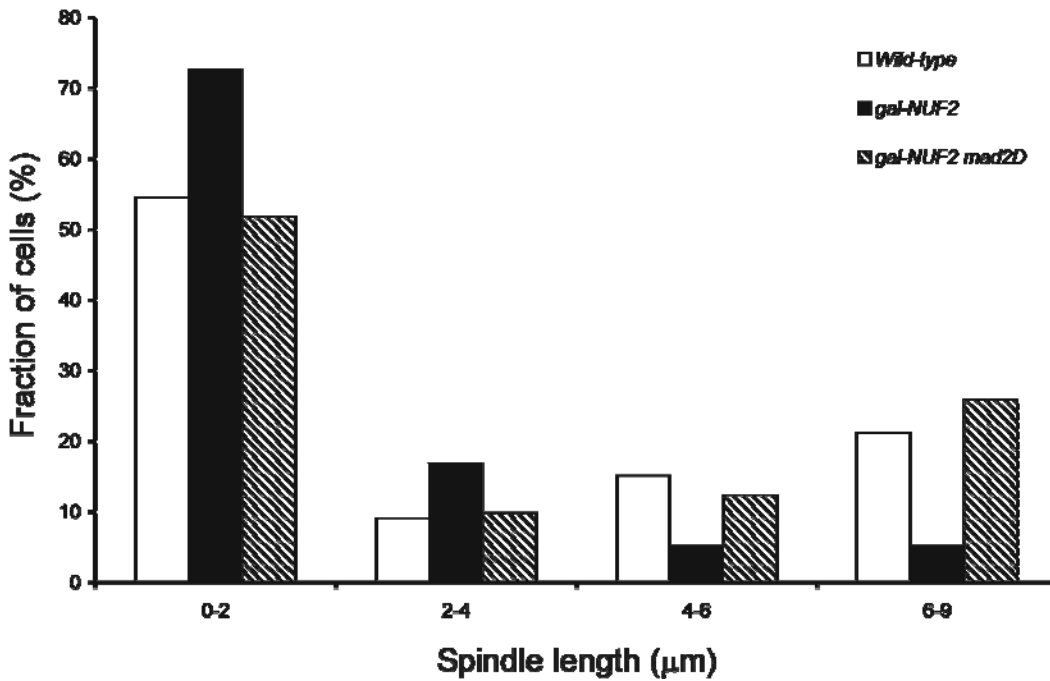


Figure 3.8: Cell separation defects in *ndc10-1* cells.

(A) *ndc10-1* cells grown at 37°C for 3-4 hours form multi-cell clusters. (B) Fluorescence images of the septin Cdc3p-GFP in wild-type and *ndc10-1* cells. In wild-type cells, Cdc3p-GFP splits into two rings at the time of cytokinesis, but in *ndc10-1* the septin ring fails to divide.

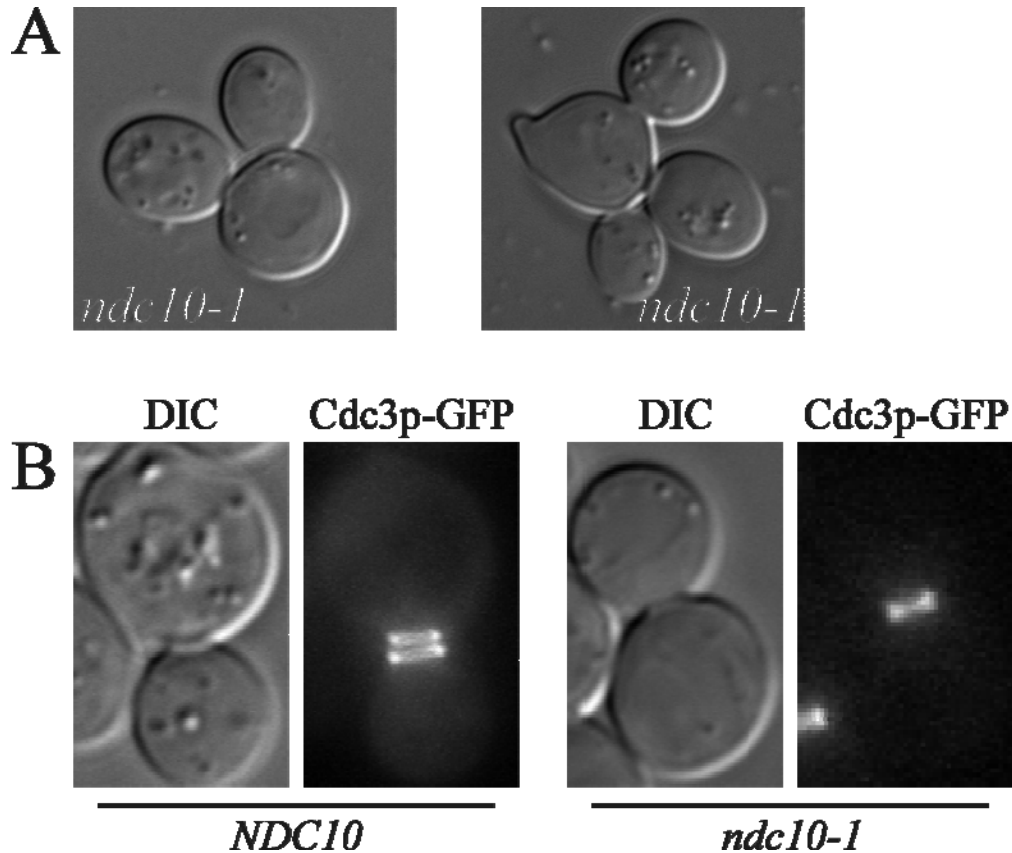
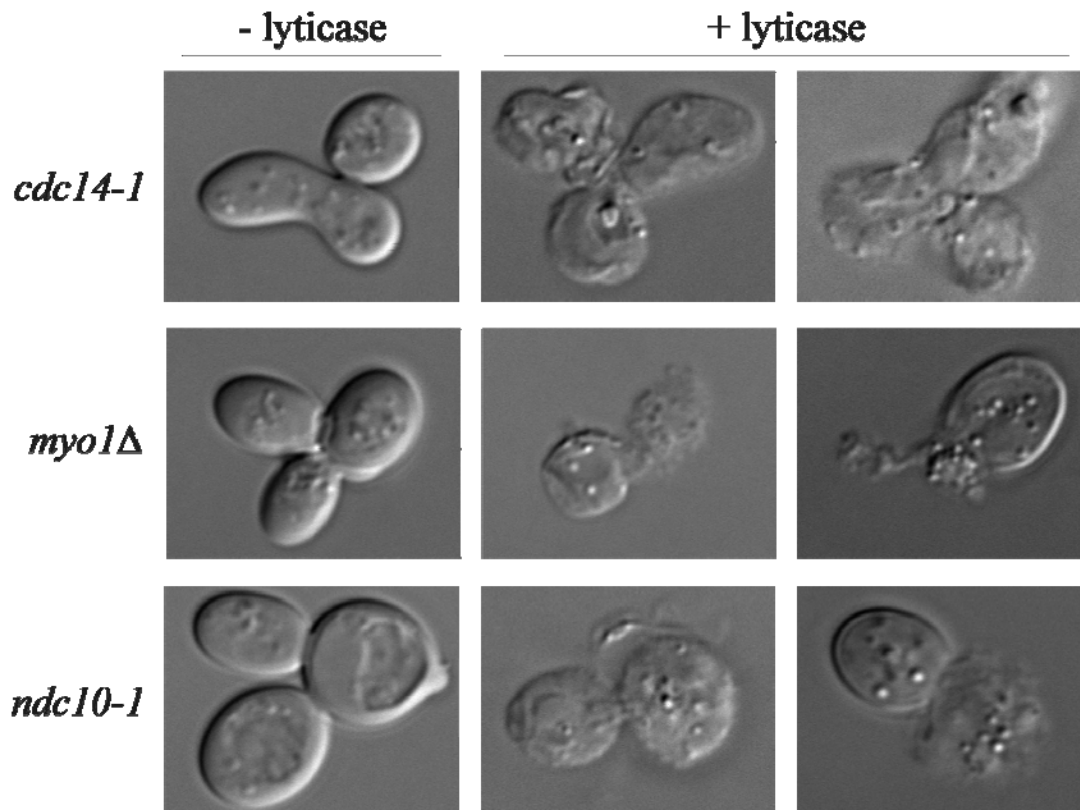


Figure 3.9: *ndc10-1* cells show cell separation defect.

cdc14-1, *myo1Δ*, and *ndc10-1* cells were treated with lyticase for 60 minutes to digest the cell wall. While the buds of *cdc14-1* cells remained attached, the buds of *myo1Δ* and *ndc10-1* cells separated.



CHAPTER FOUR

CBF3: ROLES IN SPINDLE STABILITY, CYTOKINESIS, AND KINETOCHORE ATTACHMENT

(Contributing Authors: David Bouck and Kerry Bloom)

Abstract

The spindle midzone is critical for spindle stability and cytokinesis. Chromosomal passenger proteins re-localize from chromosomes to the spindle midzone after anaphase onset. The recent localization of the inner-kinetochore, centromere-binding complex CBF3 to the spindle midzone in budding yeast has lead to the discovery of novel functions for this complex in addition to its essential role at kinetochores. In G1/S cells, CBF3 components are detected along dynamic microtubules where they can “search-and-capture” newly replicated centromeres. During anaphase, CBF3 is transported to the microtubule plus-ends of the spindle midzone. Consistent with this localization, cells containing a mutation in the CBF3 subunit Ndc10p show defects in spindle stability during anaphase. In addition, *ndc10-1* cells show defects during cytokinesis, resulting in a defect in cell abscission. These results highlight the importance of midzone-targeted proteins in coordinating mitosis with cell division. Here we discuss these findings and explore the significance of CBF3 transport to microtubule plus-ends at the spindle midzone.

Introduction

Successful cell division requires the coordination of chromosome segregation and cytokinesis. In many higher eukaryotes, this coordination is manifested in the determination of the cytokinetic furrow via mitotic spindle position (Rappaport, 1996). The proteins which localize to the spindle midzone in these cells contribute to furrow positioning and later to cytokinesis. In contrast, in many plants and fungi where the plane of division is determined prior to spindle formation, the spindle must align itself perpendicular to the division plane to ensure that equal complements of the genome will be segregated to opposite sides of the division plane (Pearson and Bloom, 2004). In these cells, proteins directed to the spindle midzone may function as a signal for the initiation of cytokinesis, thus preventing cell division until the mitotic spindle has elongated along the proper axis. Regardless of whether the spindle defines the division plane, or the cell shape defines the orientation for the spindle, the coordination of chromosome segregation and cytokinesis remains essential to the viability of the resulting daughter cells.

Among the proteins likely to couple chromosome segregation with cytokinesis are those which localize first to centromeres and later to the midzone of the anaphase spindle. The spindle midzone is comprised of overlapping microtubules plus-ends and is the future site of cytokinesis. First identified in 1987, chromosomal passenger proteins undergo programmed relocalization from chromosomes to the spindle midzone during anaphase (Cooke et al., 1987). This spatial-temporal regulation reflects the changing functions of these proteins during mitosis. Passenger proteins, including aurora B kinase, INCENP, Survivin, TD-60, Dasra B/Borealin, and Dasra A/Borealin-2, first localize to chromosomes where they function in chromatin compaction and subsequently re-localize

to the spindle midzone after anaphase onset (Adams et al., 2000; Andreassen et al., 1991; Gassmann et al., 2004; Kaitna et al., 2000; Kang et al., 2001; Leverson et al., 2002; Sampath et al., 2004; Wheatley et al., 2001). Consistent with their localization to the midzone, passenger proteins contribute to both spindle stability and cytokinesis (Andrews et al., 2003; Carmena and Earnshaw, 2003).

Homologs of the passenger proteins aurora B, survivin, and INCENP have been identified in both *Saccharomyces cerevisiae* (Ipl1p, Bir1p, and Sli15p) and *Schizosaccharomyces pombe* (Ark1, Bir1/Cut17, and Pic1). In addition to sequence conservation across eukaryotes, the functions of these proteins are well conserved. As in tissue cells, these proteins have been shown to function during chromosome segregation, spindle regulation, and cytokinesis (Biggins et al., 1999; Buvelot et al., 2003; Kang et al., 2001; Kim et al., 1999; Leverson et al., 2002; Morishita et al., 2001; Petersen et al., 2001; Uren et al., 1999; Yoon and Carbon, 1999).

In addition to forming the scaffold for Ipl1p, Bir1p, and Sli15p localization, the yeast anaphase spindle midzone hosts a number of other proteins involved in mitosis, including the spindle structural proteins Ase1p and Slk19p (Pellman et al., 1995; Schuyler et al., 2003; Sullivan et al., 2001; Zeng et al., 1999). We have recently found that the inner kinetochore complex CBF3 (centromere binding factor 3; consisting of Ndc10p, Ctf13p, Cep3p and Skp1p) is transported to the spindle midzone during anaphase, where it localizes to the plus-ends of microtubules (Bouck and Bloom, 2005). CBF3 remains associated with microtubule plus-ends during spindle disassembly, and is detected along dynamic projections emanating from the spindle pole body in G1 (Figure 4.1A).

The localization of CBF3 to growing and shortening microtubule plus-ends and the spindle midzone is the first demonstration of an inner-kinetochore, centromere-binding complex moving to the midzone (Bouck and Bloom, 2005). This finding highlights the midzone as a key site within the cell where proteins may interact as part of the signaling process essential for coordinating cellular functions. Here we discuss three possible roles for CBF3 in addition to its previously defined role in chromosome segregation. First, midzone CBF3 could contribute to anaphase spindle stability; second, midzone CBF3 could act as part of the pathway which ensures that cytokinesis follows chromosome segregation; third, microtubule plus-end associated CBF3 could form a “pre-kinetochore” structure which matures into a complete kinetochore in the subsequent cell cycle. These novel CBF3 roles are not mutually exclusive of each other and represent a means by which CBF3 may function at different points throughout the cell cycle.

Results and Discussion

Ndc10p function in anaphase spindle stability

The spindle midzone is comprised of overlapping microtubule plus-ends. In budding yeast, these microtubules are arranged with defined spacing and organization (Winey et al., 1995). As the spindle elongates during anaphase B, anti-parallel microtubules slide against each other as they polymerize, producing the force necessary to separate the spindle pole bodies. Microtubule sliding is presumably a result of plus-end directed motor proteins crosslinking anti-parallel microtubules. Failure to promote microtubule polymerization, or crosslink and slide microtubules would result in short or fragile anaphase spindles.

The spindle midzone is populated by the dynamic plus-ends of microtubules. In budding yeast, microtubules minus-ends (embedded in the spindle pole bodies) do not contribute to microtubule dynamics (Maddox et al., 2000; Tanaka et al., 2005). Growth and shortening of microtubules appear to occur exclusively at plus-ends. At the spindle midzone, factors promoting microtubule polymerization must act to promote the microtubule growth necessary for spindle elongation.

Among the proteins at the midzone, Slk19p and Sli15p have been identified as contributing to spindle stability (Pellman et al., 1995; Schuyler et al., 2003; Sullivan et al., 2001; Zeng et al., 1999). Additionally, Ipl1p plays a role in regulating the timing of spindle disassembly, as *ipl1-321* mutants spend an additional 6 minutes in anaphase prior to spindle disassembly (Buvelot et al., 2003). The mechanism by which Ipl1p regulates the timing of spindle disassembly has not been determined, however two likely

possibilities exist. Upon full spindle elongation, Ipl1p could activate a microtubule “depolymerase” at microtubule plus-ends to initiate spindle disassembly. One possibility for this microtubule depolymerase is Kar3p, which shows microtubule depolymerase activity *in vitro*, localizes weakly to spindle microtubules, and contains four putative Ipl1p phosphorylation sites based on the described consensus sequence (Cheeseman et al., 2002; Endow et al., 1994; Page et al., 1994). Alternatively, Ipl1p might regulate the timing of spindle disassembly through the destabilizing/delocalization of microtubule plus-end stabilizing proteins. Consistent with this hypothesis, levels of Ase1p and Slk19p at the midzone decrease during anaphase (Bouck and Bloom, 2005; Schuyler et al., 2003). Observation of the localization and stability of these proteins at the midzone in *ipl1* mutants would address this possibility.

CBF3 also contributes to spindle stability. Using *ndc10-1*, a temperature sensitive allele of *NDC10* known to disrupt kinetochore structure and function, as well as bypass the spindle checkpoint we examined spindle length and morphology in cells expressing GFP-Tub1p (Fraschini et al., 2001; Gardner et al., 2001; Goh and Kilmartin, 1993). Under non-permissive conditions, there is enrichment in 2-4 μm spindles in asynchronously growing cells lacking functional Ndc10p (Figure 4.2). Time-lapse microscopy revealed the cause of this enrichment: spindles repeat cycles of failed elongation during anaphase. Spindles in *ndc10-1* cells partially elongate (usually to about 5-6 μm), collapse, re-form, and then begin to elongate again (Bouck and Bloom, 2005). This phenotype was attributed to lost Ndc10p function, rather than lost kinetochore function, since cells lacking Nuf2p (another essential kinetochore protein) and Mad2p (allowing the cells to bypass the spindle checkpoint) showed no defects in

spindle elongation (Bouck and Bloom, 2005). Thus, in addition to functioning at the kinetochore, Ndc10p appears to play a role in stabilizing the spindle during anaphase elongation.

Since CBF3 has neither microtubule binding nor motor properties, it seems unlikely that CBF3 itself plays a direct role in cross-linking or stabilizing anti-parallel microtubules at the spindle midzone (Sorger et al., 1994). As part of the kinetochore, CBF3 serves as a foundation or scaffold for assembly of the remainder of the kinetochore (McAinsh et al., 2003). Likewise, at the spindle midzone, CBF3 might aid the assembly of microtubule associated proteins directly responsible for spindle stability. The organization and dynamics of these proteins (Ase1p, Slk19p, and Sli15p) in *ndc10-1* cells could shed light on this possibility.

Coordination of chromosome segregation with cell division

In most eukaryotes, nuclear envelope breakdown and chromosome condensation mark entry into mitosis. Dissolution of the nuclear envelope also enables interaction between the mitotic machinery and the cell cortex, thus enabling the orchestration of chromosome segregation and cytokinesis.

For many years, the formation of a midbody complex appeared to be essential for the positioning of the cytokinetic furrow and completion of cytokinesis, however more recently it has been shown that cells lacking a bipolar spindle (and thus a spindle midzone/midbody) are able to furrow and complete cytokinesis following an induced anaphase (Canman et al., 2003). In these cells, the stable attachments of microtubules to cortical regions may allow for the delivery of factors necessary for completion of cell

division. This model allows for the coordination of the mitotic and cytokinetic machineries through spatial regulation and delivery of proteins. Thus, the delivery of certain components to microtubule plus-ends, rather than delivery of these components to the midbody, might be the critical signaling event for cytokinesis.

In budding yeast the nucleus remains intact during mitosis. Wild-type budding yeast form binucleates and anucleates (a result of undergoing cytokinesis prior to either proper spindle alignment or spindle elongation) extremely rarely, and even mutants affecting spindle positioning show < 5% binucleate cells (Li et al., 1993). This suggests that budding yeast have a mechanism by which the mitotic spindle signals to the bud neck for initiation of cytokinesis.

We have found that a previously unidentified phenotype of *ndc10-1* cells is the formation of multi-cell clusters (Figure 4.3). This morphology can be a result of either failure to complete cytokinesis, or failure in cell abscission following cytokinesis. FLIP (Fluorescence Loss In Photobleaching) analysis of cytoplasmic GFP expressed in *ndc10-1* multi-budded cells showed that these cells successfully complete cytokinesis, but fail to separate from each other following cytokinesis (Figure 4.3). When the mother cell was specifically photobleached, fluorescence was lost in only one of the two attached daughters. This suggests that the bud that retained fluorescence had completed cytokinesis, while the other bud still shared a continuous cytoplasm with the mother cell. Cell wall digestion of fixed, multi-cell clusters also showed that these cells had completed cytokinesis, but failed to separate from each other (Bouck and Bloom, 2005).

To better understand the basis of this failure in cell separation, the localization and organization of septins were examined in *ndc10-1* cells. In wild-type cells, septins

form a ring at the bud neck, which splits into two rings around the time of cytokinesis (Lippincott et al., 2001). Septins have been proposed to recruit or retain factors to the bud neck essential for cytokinesis and cell separation (Dobbelaere and Barral, 2004). In *ndc10-1* cells, the septin ring fails to split into two rings (Bouck and Bloom, 2005). This defect likely causes the disorganization/delocalization of components needed at the neck during cytokinesis and cell separation.

How might the kinetochore communicate with the cytokinetic machinery? We can imagine two plausible means by which the kinetochore signals to the mother-bud neck in a closed mitosis. First, signaling through the spindle pole body. Spindle pole bodies nucleate microtubules to both the nucleus and cytoplasm. These microtubules may act as signaling conduits emanating from a central hub, the spindle pole. During anaphase, centromere-kinetochore complexes are drawn close to the nuclear side of the spindle pole body, and during spindle disassembly many passenger proteins (including Ipl1p, Sli15, Ndc10p, Cep3p) reel back to the spindle pole bodies on depolymerizing microtubule plus-ends (Bouck and Bloom, 2005; Buvelot et al., 2003). Either of these events could act as a signal transduced from the nuclear face to the cytoplasmic face of the spindle pole body. This signal could then be carried to the neck by cytoplasmic microtubules or diffusion of signaling molecules from the cytoplasmic face of the spindle pole body.

Alternatively, the delivery of proteins to microtubule plus-ends at the midzone during late anaphase could be the initiating step in signaling to the neck that chromosomes have segregated. The spindle midzone is located in close proximity to the mother-bud neck. Formation of the midzone complex could cause a signal to the neck

through steric interaction of the nuclear envelope with the neck cortex, or through signaling through nuclear pores located near the midzone. Interestingly, many spindle checkpoint proteins have been localized to the nuclear pore complexes, and spindle checkpoint mutants also form multi-cell clusters when treated with microtubule depolymerizing agents (Hoyt et al., 1991; Iouk et al., 2002; Li and Murray, 1991). Together, these data suggest that failure to form a midzone complex (whether caused by spindle fragility in *ndc10-1* cells, or microtubule poisons) leads to a failure to complete cell division.

Building a kinetochore

One of the greatest questions remaining in the field of mitosis research is how a centromere-kinetochore-microtubule attachment is formed. Three possibilities exist: first, a kinetochore can be built on a centromere and wait for a dynamic microtubule to find it. Second, a kinetochore could form on a microtubule and search for a centromere. Third, part of the kinetochore could form on the centromere and part on a microtubule; when these complementary complexes find each other a kinetochore is formed.

Discoveries of the last few years suggest that the kinetochore might be divided up into microtubule-associated and centromere-associated complexes. The outer kinetochore Dam1 complex has recently been shown to form rings around microtubules *in vitro* (Miranda et al., 2005a; Westermann et al., 2005). This structure, if present *in vivo*, would likely remain associated with microtubules despite the presence or absence of centromeric DNA. In fact, the interface between the Dam1 ring complex and the Ndc80 complex appears to be the site of detachment/reattachment as mediated by the Ip11p

kinase (Shang et al., 2003). Since other kinetochore proteins and complexes, including the CBF3 complex, Cbf1p and Cse4p associate directly with centromeric DNA, it seems likely that the kinetochore is made up of complexes associated more directly with either the centromere or microtubules.

While increased understanding of kinetochore structure yields insights into how the subcomplexes of the kinetochore are arranged with respect to each other, it fails to directly address the question of how the centromere-kinetochore-microtubule structure is formed. The recent localization of CBF3 components to the plus-ends of growing and shrinking microtubules following spindle disassembly suggests that this complex may function in the next cell cycle in a “search-and-capture” mechanism to establish new kinetochore attachments (Bouck and Bloom, 2005; Tanaka et al., 2005). Since the CBF3 complex specifically binds centromeric DNA, and has now been localized with dynamic microtubules, this “pre-kinetochore” complex is capable of tethering CEN-bound chromosomes to microtubules (Figure 4.1A). Upon CBF3 binding of a centromere, additional kinetochore proteins and complexes could be recruited to this structure in a process of kinetochore maturation. The centromere-kinetochore-microtubule attachment has to be strong enough to withstand forces exerted during metaphase, and the quality of attachments has to be monitored by the spindle checkpoint. The addition of these additional complexes might offer increased stability and checkpoint function to the kinetochore.

Conclusion

While microtubules have long been appreciated for their roles in nuclear positioning, nuclear orientation, and chromosome segregation, the formation of the spindle midzone and the delivery of proteins to microtubule plus-ends appear to be additional essential microtubule functions. Proteins associated with the spindle midzone are optimally positioned to act in the coordination of chromosome segregation and cytokinesis due to their dissociation from chromosomes after anaphase onset and their localization at the plane of division. We have found that the inner kinetochore complex CBF3 is targeted to the spindle midzone during anaphase, and that it remains associated with dynamic nuclear microtubules in G1/S cells. CBF3 association with microtubule plus ends could facilitate search-and-capture of centromeres following centromere replication in early S phase (Figure 4.4). Once kinetochores are formed, CBF3 is essential to kinetochore function (i.e. tethering chromosomes to dynamic microtubule plus-ends). During anaphase, CBF3 is found along the interpolar microtubules and may function to stabilize the elongating spindle. CBF3 is enriched at the spindle midzone in late anaphase, where it might function in signaling the completion of chromosome segregation. These functions show that CBF3 may contribute to kinetochore assembly, spindle stability, and the coordination of chromosome segregation with cytokinesis.

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Figures

Figure 4.1: Non-centromeric localization of Ndc10p-YFP along microtubules.

Asynchronously growing cells expressing Cse4p-CFP (centromere-specific histone H3 variant) and Ndc10p-YFP (pseudocolored as red and green, respectively) were imaged at various cell cycle stages by fluorescence microscopy. (A) In unbudded cell, Cse4p appears as a single spot, representing clustered centromeres (solid arrow), while Ndc10p is also detected along a projection extending from the cluster (hollow arrow). (B) During metaphase, Cse4p and Ndc10p co-localize to two cluster representing bioriented sister chromatids. (C) In anaphase, Ndc10p localizes to the interpolar microtubules and spindle midzone (hollow arrow), while Cse4p appears as two discrete clusters.

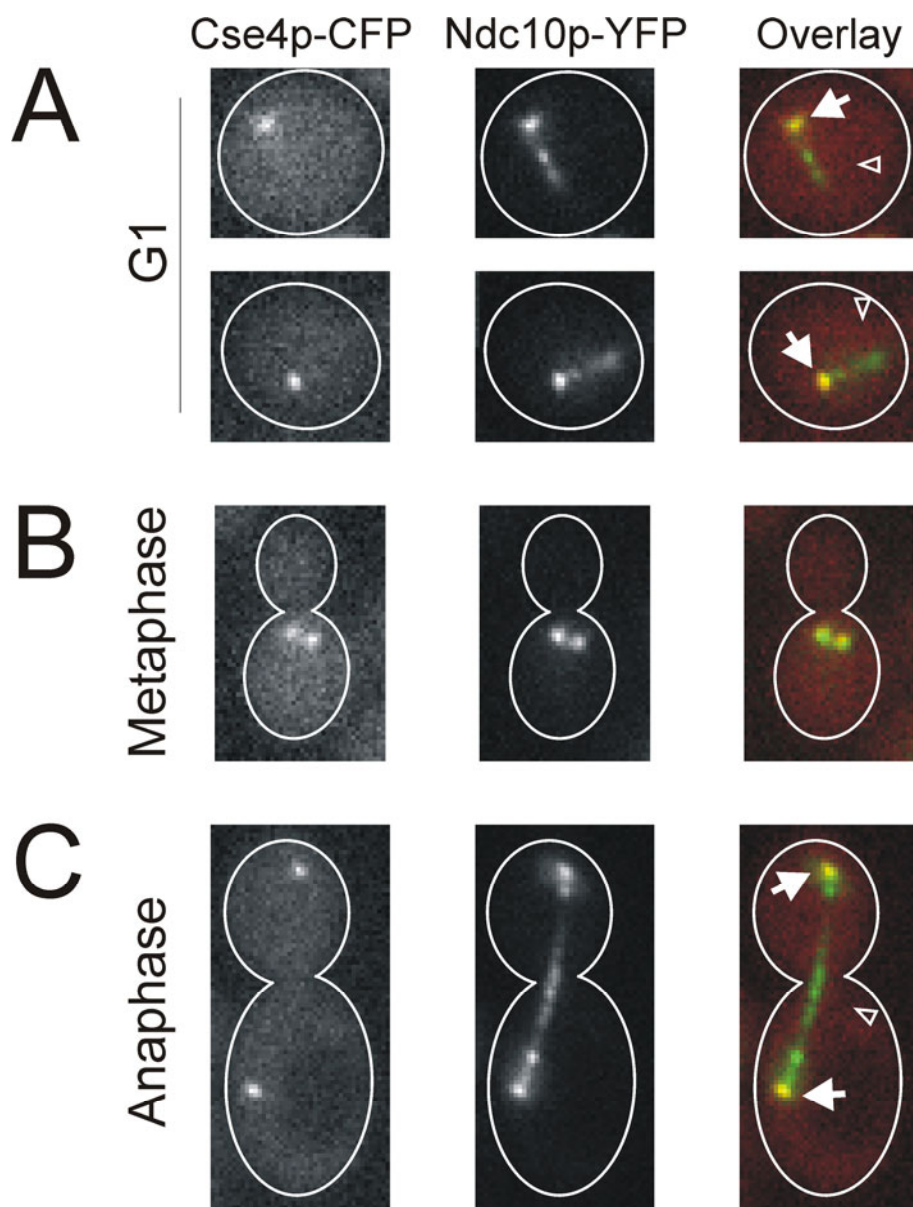


Figure 4.2: Spindle elongation defect in cells lacking functional Ndc10p.

Asynchronously growing *ndc10-1* cells were shifted to non-permissive conditions for 90 min and then spindle length was measured using GFP-Tub1p. *ndc10-1* cells showed an enrichment of shorter spindles, indicating a defect in spindle elongation.

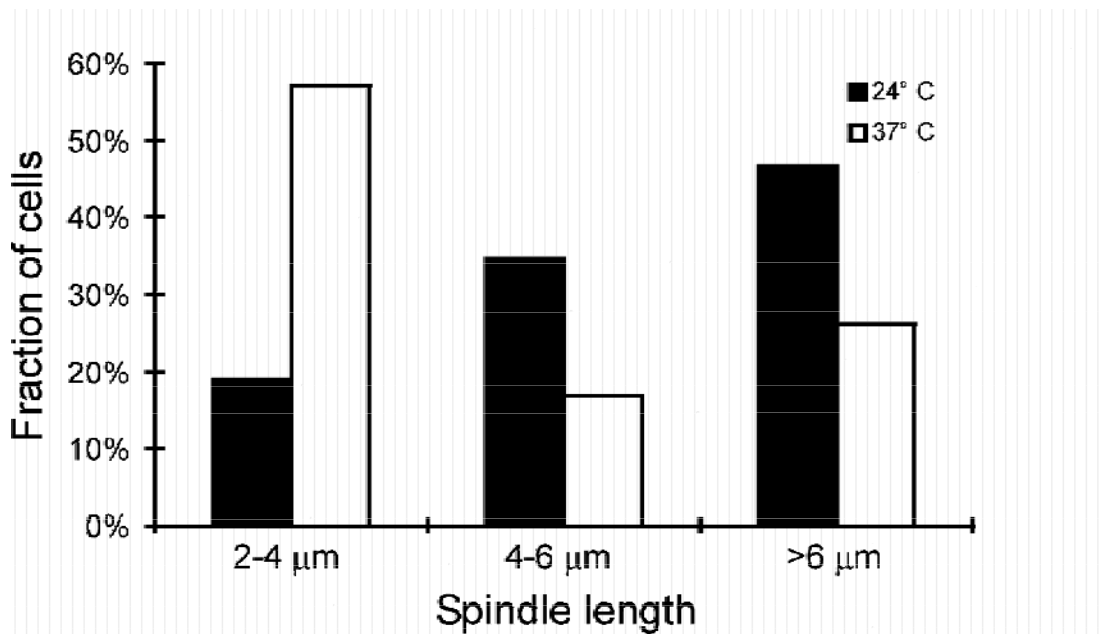


Figure 4.3: Multi-cell clusters in *ndc10-1* mutants caused by cell separation defect.

ndc10-1 cells expressing cytoplasmic GFP were shifted to non-permissive temperature for 3 hours. GFP within the mother was specifically photobleached with 4 laser (488nm) exposures. Fluorescence intensity measurement of the mother and both buds showed partitioning between the mother and Bud 1, indicating that cytokinesis had completed by cell separation was defective.

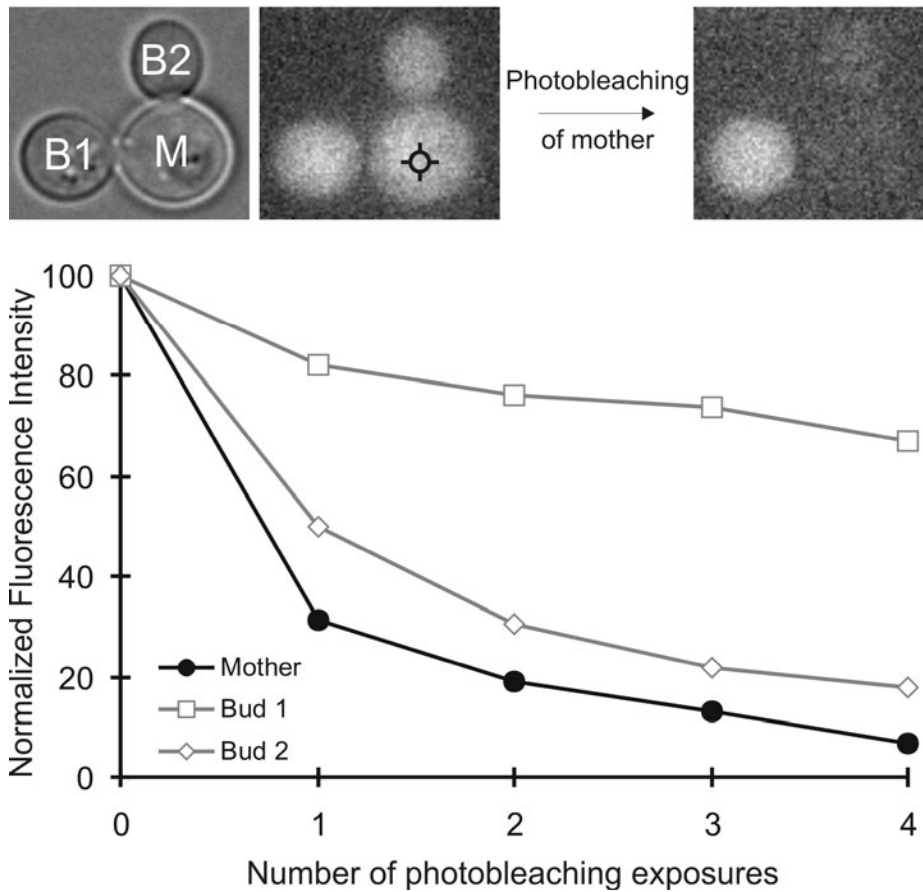
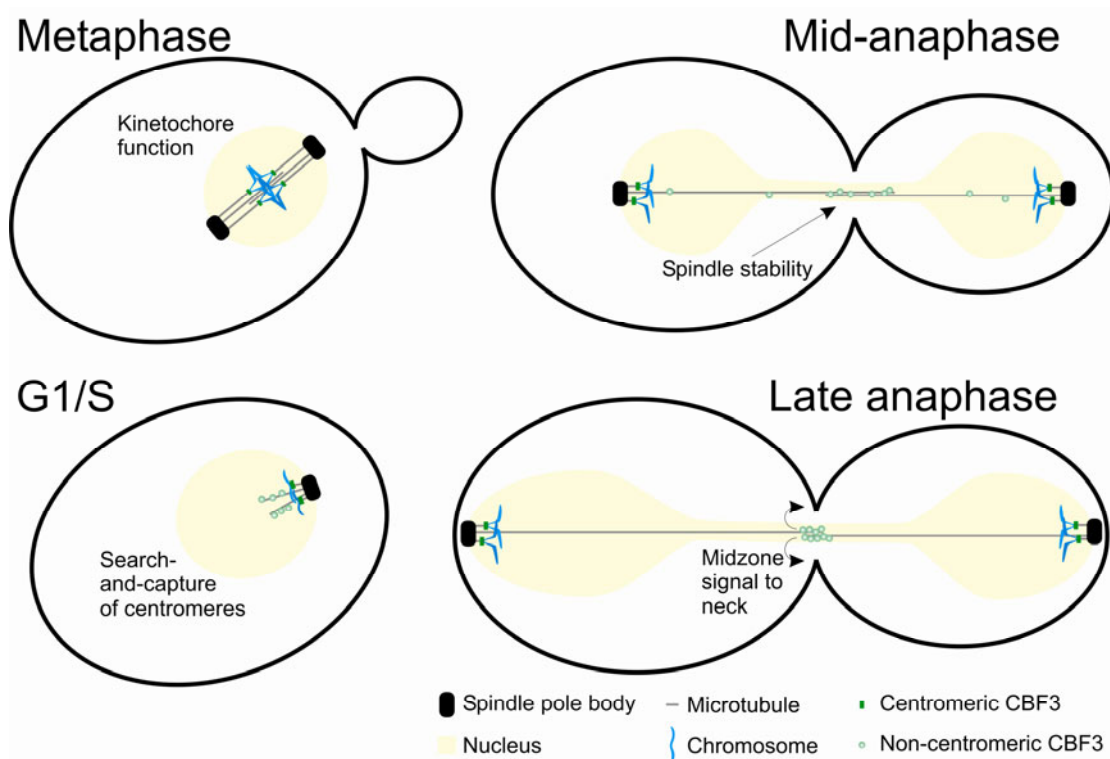


Figure 4.4: Model of the changing roles of CBF3.

During G1/S, CBF3 may be involved in a search-and-capture mechanism for the capture of centromeres. Once kinetochores are formed CBF3 is essential for kinetochore function. During anaphase, CBF3 localizes to the midzone where it might play a role in stabilizing the anaphase spindle. Once the spindle has fully elongated, CBF3 is enriched at the midzone, where it could signal to the cytokinesis machinery at the mother-bud neck.



CHAPTER FIVE

SUMMARY AND CONCLUSIONS

Summary

Mitosis is among the longest studied biological processes, yet the present time is one of the most exciting for new insights into how this amazingly accurate process is carried out in a variety of organisms. In these studies, the budding yeast *Saccharomyces cerevisiae* has been used because of its rapid cell cycle and ease of genetic manipulation. Additionally, the proteins and individual phases of mitosis are well conserved across eukaryotes, suggesting that these findings may be applicable to research in other organisms. Taken together with the relative simplicity of the yeast spindle, kinetochore, and centromere, budding yeast becomes a powerful model organism with which to increase our understanding of mitosis.

The first chapter of this dissertation reviewed the key components of the spindle and the function of each component. The understanding of these proteins, protein complexes, and polymers has grown rapidly during recent years due to the *molecular revolution* in biological research. The sequencing of genomes and ever-growing set of molecular tools have facilitated the reduction of cellular process to the level of individual proteins and their interactions. Indeed, much research focuses on the specific amino acid residues subjected to post-translational modification, necessary for binding to a partner protein, or critical for enzymatic activity.

The combination of these tools and the information garnered through this reductionist approach with live-cell microscopy allows for the understanding of the cellular significance of intra- and intermolecular interactions. In this work, the fusion of proteins of interest with GFP, and its variants, has proven invaluable for the study of protein localization, translocation, binding dynamics, and changes in cytoskeletal structures. In coming years, the continued combination of advanced molecular and imaging approaches to biological questions should unlock many of the remaining questions in cell biology.

The mechanics of the spindle are of particular interest since spindle integrity is essential to successful segregation of the genome. In budding yeast, kinesin motors have been the primary target of spindle mechanics investigation. The directionality and microtubule cross-linking functions of these proteins lead to an elegant model for spindle length determination and spindle stability during metaphase. In chapter two, I have tested the possible function of another component of the spindle, the chromatin packaging of sister chromatids. By lowering the concentration of nucleosomes in cells, chromatin becomes less compacted. This change in chromatin structure lead to increased sister centromere separation and increased spindle length. This result demonstrates the biophysical role of chromatin packaging in regulating spindle structure. The combination of histone repression with motor deletions further demonstrated that the stretching of chromatin exerts a force in direct opposition to the BimC kinesins, Cin8p and Kip1p. This study demonstrates the power in combining molecular tools (*e.g.* promoter replacement, GFP fusions, and gene deletions) with quantitative imaging to understand a biophysical question in the field of chromosome segregation.

Chapter three of this work begins with the temporal-spatial regulation of various kinetochore proteins throughout mitosis. The non-centromeric localization of kinetochore proteins suggests that these proteins play additional roles in the cell, and differences in localization may reveal differences in these roles. The localization of Slk19p and Ndc10p were examined further due to the temporal difference in their re-localization from kinetochores to the spindle midzone during anaphase. Slk19p localizes to the midzone immediately following anaphase onset, but does not persist at the midzone as anaphase proceeds. In contrast, Ndc10p localization to the midzone increases into late anaphase. The inverse temporal relationship in localization reflects the changes in protein composition at the spindle midzone.

Ndc10p is not alone in its localization to the spindle midzone in late anaphase. Cep3p, another CBF3 subunit, also localizes to the spindle midzone, suggesting that the entire CBF3 complex follows this pattern. CBF3 is primarily known as the centromere-specific binding complex of the kinetochore. The centromere-independent association of CBF3 with microtubules was a novel and unexpected result. This complex may reflect a “pre-kinetochore” complex that matures into a mature kinetochore upon centromere binding and the association of the remaining kinetochore proteins.

I have also identified novel, non-kinetochore phenotypes for the temperature sensitive allele, *ndc10-1*. As described in chapters three and four, *ndc10-1* cells have defects in spindle elongation and cell division. This phenotype has not been reported for other kinetochore mutants, suggesting that this defect is Ndc10p specific and not a non-specific consequence of altered kinetochore function. The cell division defect in *ndc10-1* cells is revealed as a deficiency in the latter half of cell division, cell abscission. FLIP of

soluble GFP in living cells and cell wall digestion of fixed cells both demonstrated that *ndc10-1* cells successfully complete cytokinesis (two separate cytoplasmic compartments are formed), but are unable to separate. It is possible that this phenotype is a result of errors during cytokinesis. Evidence for this idea include the finding that *myo1Δ* cells also complete a defective cytokinesis that results in a cell division defect phenotype. Additionally, septin rings fail to split during cytokinesis in *ndc10-1* cells, indicating that cytokinesis is not executed precisely like wild-type cells.

Together, these results suggest non-kinetochore roles for Ndc10p and possibly the entire CBF3 complex. The dynamic re-localization of Ndc10p during mitosis may reflect a mechanism for coordinating these steps of mitosis.

Future Directions

Since the publication of chapter three of this dissertation, other groups have pursued further understanding of the regulation of Ndc10p along the spindle, and have attempted to elucidate more details of this pathway. Ndc10p localization to the spindle is Bir1p dependent, and requires the phosphorylation of Bir1p (Widlund et al., 2006). Mutation of these phosphorylation sites resulted in spindle elongation defects, but no cytokinesis or cell separation defects were observed. Additionally, Ndc10p localization to the midzone requires the sumoylation of Ndc10p (Montpetit et al., 2006). Mutation of the mapped sumoylation sites blocked Ndc10p localization to the spindle and caused abnormal anaphase spindle morphology, but did not result in cell division errors. These findings suggest that the localization of Ndc10p to the midzone is more important for spindle integrity during anaphase than signaling to the midzone during cell division. A

separate study reports that spindle integrity during anaphase is required for proper cytokinesis and cell division through a pathway requiring Ipl1p activity (Norden et al., 2006). This conclusion is consistent with the cell division defects seen in *ndc10-1* cells and spindle checkpoint mutants that have been treated with nocodazole (Hoyt et al., 1991; Li and Murray, 1991). In both cases, spindle integrity is insulted and multi-budded cells are produced. It is unclear how this pathway interacts with the phosphorylation of Bir1p or the sumoylation of Ndc10p. Further study into the interactions between these pathways and mechanisms should prove to be an interesting area of study. Additionally, there are many other kinetochore proteins which localize to non-kinetochore structures during mitosis. Further study into their functions at these sites should contribute to the understanding of the coordination of mitotic events.

The biophysical study of chromatin and its role in the spindle will likely be an area of active investigation in the Bloom laboratory for years to come. The combination of experimental biology with computer simulations of the spindle has been a powerful means to understand the spindle (Gardner et al., 2005; Pearson et al., 2004). An increased understanding of the *in vivo* biophysical properties of chromatin will provide an additional parameter to include in computer simulations of spindle function. Thus, the biophysical characterization of chromatin in living cells will be a prime area of research. Additionally, the investigation of chromosome segregation defects in cells carrying chromatin modification mutants would provide additional support for the importance of chromatin structure in spindle function.

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